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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVII — *SEPTEMBER 1958* — NUMBER 9

Kinetics of the Degradation of Isoamyl Nitrite in Ampuls I.*

Nature of Reaction Products and Influence of Temperature, Oxygen, Alcohol, Water, and Acid on the Overall Rate

By MARTIN H. YUNKER, DALE SZULCZEWSKI, and TAKERU HIGUCHI

Results of a kinetics study on the rate of degradation of both laboratory and commercial lots of isoamyl nitrite in sealed ampuls are presented. The reaction appears to be autocatalytic with an effective heat of activation of 20 kcal./mole. Commercial ampuls containing roughly 0.33 ml. of the ester explode on developing from 7–12 ml. of by-product gases. Mass spectrometric and gas chromatographic analyses show that these consist largely of nitrogen, nitrous oxide, carbon dioxide, and traces of nitric oxide and carbon monoxide. Results obtained suggest that the first three gases are produced by a secondary reaction between nitric oxide and aldehyde. The liquid products appear to be a mixture consisting of at least twelve components including carboxylic ester, carboxylic acid, isoamyl alcohol, aldehyde, and isoamyl nitrate. The influences of water, acid, alcohol, oxygen, and certain additives on the overall degradative rate are also given.

THE PRESENT STUDY is concerned with the results of an investigation on the problem of deterioration of isoamyl nitrite (amyl nitrite, U. S. P.) on storage. The nitrite ester on standing undergoes spontaneous decomposition, yielding both gaseous and liquid degradation products. This instability is manifested in commercial amyl nitrite ampuls by a steady increase in internal pressure leading eventually to explosive destruction of the glass container. In this work, experimental data were obtained showing the chemical nature of the end products, the rate of pressure development, the influence of temperature, water, and oxygen on the rate, and finally the catalytic effect of acid for the liquid phase, thermal breakdown reaction of the ester especially in sealed ampuls.

Much of the past studies on the decomposition reactions of the nitrite esters have dealt with vapor phase, high temperature systems.

The thermal decomposition of methyl, ethyl, *n*-propyl, isopropyl, and *n*-butyl nitrites in the vapor phase has been studied by Steacie and coworkers (1) at 170–200°. Under these conditions the major overall reaction in each case appeared to be:

$$2RCH_2ONO \rightarrow RCHO + RCH_2OH + 2NO$$

Rice and Rodowskas (2) have proposed the following mechanism for the vapor phase reaction of alkyl nitrites:

$$RCH_2ONO \rightarrow RCH_2O\cdot + NO$$
$$RCH_2O\cdot + RCH_2ONO \rightarrow RCH_2OH + R\dot{C}HONO$$
$$R\dot{C}HONO \rightarrow NO + RCHO$$

In a more recent study of the vapor phase reaction of ethyl nitrite, however, Levy (3) disagrees with Rice's mechanism, and finds that the following scheme agrees better with his kinetic data.

* Received February 13, 1958, from the School of Pharmacy, University of Wisconsin, Madison.

$$C_2H_5ONO \rightarrow C_2H_5O\cdot + NO$$
$$C_2H_5O\cdot + NO \rightarrow CH_3CHO + NOH$$
$$2NOH \rightarrow N_2O + H_2O$$
$$C_2H_5O\cdot + NHO \rightarrow C_2H_5OH + CH_3CO$$
$$NOH + CH_3CO \rightarrow CH_3CHO + NO$$

In each of the above studies, nitric oxide was found to be the principal gaseous product while alcohol and aldehyde were the predominant liquid decomposition products. In addition, however, Levy found appreciable nitrous oxide, and had reason to believe water is formed.

The only study of the liquid phase decomposition of a nitrite ester was made by Kornblum and Oliveto (4) on 2-octyl nitrite at 100°. In this study, the authors continually flushed the reacting system with carbon dioxide and collected the gaseous products above 50 per cent potassium hydroxide. The principal products of the reaction were found to be nitric oxide, 2-octanone, and 2-octanol as would be expected by the previously postulated mechanisms for the vapor phase reaction. In addition, they found significant amounts of nitrogen, nitrous oxide, and carbon dioxide as gaseous products, and several acids and esters of various chain lengths as liquid products. The formation of these additional compounds was thought to arise from the cleavage of 2-octanone by nitric oxide followed by esterification of 2-octanol by the acids formed.

CHEMICAL NATURE OF PRODUCTS

There seems to have been no earlier attempts made to establish the chemical nature of the products resulting from decomposition of isoamyl nitrite in ampuls. As a phase of the present investigation, both the liquid and the gaseous products formed during the breakdown of the ester in sealed ampuls were analyzed.

Gaseous Products.—Gases isolated from commercial ampuls of the ester ranging in age from two to four years were analyzed qualitatively and quantitatively by both mass spectrometry and by gas chromatography. A typical analysis using gas chromatography (5) of the gaseous phase found in old amyl nitrite ampuls (Burroughs Wellcome & Co., Lot 143, date of manufacture November 26, 1951) is shown in Table I. The accuracy of the method is indicated by the data in Table II which gives the results of analysis of a mixture of similar gases of known composition. From this it is seen that the gas phase consisted of a mixture of nitrogen, nitrous oxide, carbon dioxide, and traces of nitric oxide and carbon monoxide. The presence of the large amount of nitrogen and the near absence of nitric oxide were rather surprising in view of the results obtained by earlier workers. No indication of any oxygen was found despite the fact that the ampuls were sealed under air. Because of this apparent ambiguity several experiments were conducted to illustrate possible routes by which nitric oxide could disappear from the system with the production of nitrogen and the other significant nitrogen containing gas, nitrous oxide.

The observation of Kornblum and Oliveto (4) that nitric oxide is capable of reacting with 2-octanone at 100° to yield liquid oxidation products and nitrogen, nitrous oxide, and carbon dioxide led the authors to investigate the possibility of a similar reaction with an aldehyde at a lower temperature. The results of analyzing the gas phase obtained from the reaction of *n*-butyraldehyde and nitric oxide at 50° are seen in Table III. From these data it is seen that in only one case was there evidence of a reaction. In this case the resulting gaseous products were similar to those observed to be present in old amyl nitrite ampuls.

TABLE I.—PERCENTAGE COMPOSITION OF GAS FOUND IN OLD AMYL NITRITE AMPULS[a]

Sample	N_2	N_2O	CO_2	NO	CO
1	50	33	15	Trace	Trace
2	48	35	12	Trace	Trace
3	48	33	19	Trace	Trace
4	48	34	16	Trace	Trace
5	49	37	14	Trace	Trace
Av.	49[b]	34[b]	15[b]		

[a] Burroughs Wellcome & Co., Lot 143, date of manufacture November 26, 1951.
[b] Refers to per cent by volume.

TABLE II.—ANALYSIS OF A GASEOUS MIXTURE OF KNOWN COMPOSITION BY GAS CHROMATOGRAPHY (5)

	%			
Gas	O_2	N_2	N_2O	CO_2
Known	19	38	23	19
Found[a]	17	37	24	21

[a] Average of five determinations.

TABLE III.—ANALYSIS OF THE GAS PHASE RESULTING FROM REACTION OF NITRIC OXIDE AND *n*-BUTYRALDEHYDE AT 50°

	%			
Time, Hr.	N_2	NO	N_2O	CO_2
0	2	98	0	0
24.5	2	98	0	0
47.5	67	0	22	11
96.0	2	98	0	0
211.0	8	92	0	0
288.0	6	94	0	0

Wt. *n*-butyraldehyde, 22 mg.; volume ampul, 2.5 ml.; pressure filled under, 74 cm. Hg; temperature filled under, 25°.

Since the behavior of this reaction with time is ambiguous, it was thought possible that some catalytic species was inadvertently introduced into the particular ampul which showed complete destruction of nitric oxide. Since contamination by a small quantity of air would not be unlikely, another experiment was performed in identical manner as before with the exception that the nitric oxide was deliberately contaminated with oxygen. As seen from Table IV in the presence of even the smallest concentration of oxygen, the nitric oxide reacts with the aldehyde completely in the relatively short incubation time of twelve hours. In all probability the catalytic species is either nitrogen dioxide or its dimer. This observation is substantiated by the

TABLE IV.—ANALYSIS OF THE GAS PHASE RESULTING FROM THE REACTION OF NITRIC OXIDE WITH A TRACE OF NITROGEN DIOXIDE AND *n*-BUTYRALDEHYDE AT 50°

Time, Hr.	O_2 Added	% N_2	NO	N_2O	CO_2
0	0	2	98	0	0
12	2	68	0	20	12
12	4	64	0	25	11

Wt. *n*-butyraldehyde, 22 mg.; volume ampul, 2.5 ml.; pressure filled under, 74 cm. Hg; and temperature filled under, 25°.

work of Kuhn (7) who observed a similar phenomenon in the case of nitric oxide oxidation of benzaldehyde.

The ability of nitric oxide to react with aldehyde, a reducing agent, prompted investigation into another possible nitrogen producing reaction, namely that of nitric oxide and the ester itself. By referring to Table V one sees that it appears as if nitric oxide will react with isoamyl nitrite itself under these conditions with the production of nitrogen, nitrous oxide, and carbon dioxide. These results are not entirely clear due to the likely presence of an aldehyde. However, it is felt that the production of nitrogen is too rapid to be explained entirely on the basis of a possible aldehyde-nitric oxide reaction.

In order to ascertain whether nitric oxide would be found in a significant amount if the gaseous products were continually removed from the reacting system, an experiment similar to that of Kornblum and Oliveto was performed. The results are summarized in Table VI. The presence of potassium hydroxide precluded any analysis for carbon dioxide. The increase of nitric oxide from the trace found in the case of the closed system to 40%, as indicated, is significant.

The possibility of nitrous oxide, by virtue of its relatively low decomposition temperature and the exothermic nature of its decomposition, acting as a nitrogen producing intermediate was investigated.

TABLE V.—ANALYSIS OF THE GAS PHASE RESULTING FROM THE REACTION OF ISOAMYL NITRITE AND NITRIC OXIDE AT 50°

Time, Hr.	% N_2	NO	N_2O	CO_2
0	2	98	0	0
23	12	86	0	2
51	22	72	1	5
72.5	37	59	< 1%	3
120.0	65	< 1%	12	18
190.0	70	Trace	14	16

Ampul volume, 2.5 ml.; wt. isoamyl nitrite, 35 mg.; temperature at filling, 24°, and pressure filled at, 74 cm. Hg.

TABLE VI.—ANALYSIS OF THE GAS PHASE RESULTING FROM DEGRADATION OF 100 ML. OF ISOAMYL NITRITE AT 70° IN REACTING SYSTEM CONTINUALLY FLUSHED WITH CARBON DIOXIDE. GASEOUS PRODUCTS COLLECTED ABOVE 50 PER CENT POTASSIUM HYDROXIDE

Time, hr.	48
Nitrogen, %	58
Nitric Oxide, %	40
Nitrous Oxide, %	1

Ampuls containing 35 mg. of isoamyl nitrite and filled under 76 cm. Hg pressure of nitrous oxide were incubated for 240 hours at 50°. Two additional experiments were conducted in which the isoamyl nitrite was replaced with isoamyl alcohol and *n*-butyraldehyde. In each case the results indicated that no reaction occurred.

Liquid Products.—Infrared absorption spectra of the liquid phase isolated from ampuls which were kept at 70° over a two week period showed a constant increase in carbonyl and organic nitrate absorption. During the first seven or eight days of storage, two peaks were present in the carbonyl region at 5.8μ and 5.86μ, but only a single peak at 5.82μ was present after two weeks. This would indicate that a transformation from acid to ester was occurring during the decomposition. The addition of oxygen was found to considerably increase the amount of nitrate and acid formed during the first several days of storage.

Infrared absorption spectra of the fraction obtained from a fractional distillation of a sample kept for two weeks at 80° showed ester to be the predominant product, with acid, aldehyde, alcohol, and nitrate also present in lesser amounts. Characterization of the low and high boiling fractions by chemical means identified isoamyl isovalerate, isovaleryl aldehyde, and isovaleric acid as being present in the liquid mixture. Water, which was present as an insoluble layer in the original decomposition mixture, accounted for approximately 8–10% of the total products.

As previously mentioned, an experiment in which the ester was allowed to decompose in an open, flushed system was conducted. Infrared absorption spectra of samples from this experiment collected over a twelve day period of storage at 70° showed that no significant degradation had occurred. This would indicate that the mechanism proposed by Steacie is not primarily responsible for the products of the degradation of isoamyl nitrite in the liquid phase in sealed ampuls.

A more thorough study based on vapor phase chromatographic separation of the reaction products has shown that at least 12 products are formed in the liquid phase. The results of this investigation will be published shortly.

INFLUENCE OF TEMPERATURE

The temperature dependency of the reaction was studied over the range of 50–80°. Figure 1 shows the increase in gas volume per ampul to be linear with time over the range studied. The measurements were carried out on commercially prepared ampuls which were approximately one year old. The amount of gas present in the ampuls at the beginning of the experiment varied from 4 to 6 cc., consisting largely of gases produced during storage. This volume of gas, representing from 15 to 20% degradation, probably corresponded to the rapid phase of the reaction. This part of the reaction is responsible for the final, linear portion of the curve shown in Fig. 4 for ampuls filled under air. The average 10° temperature coefficient corresponds to approximately 2.4. The average heat of activation as determined from the Arrhenius plot (Fig. 2) for this portion of the degradative reaction was 20 kcal./mole.

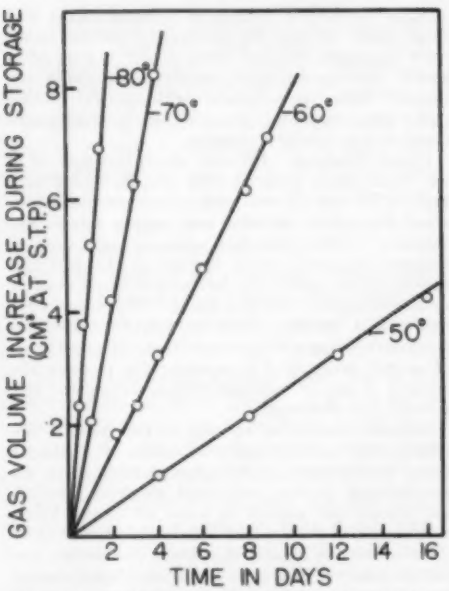

Fig. 1.—A plot showing the effect of temperature on the rate of gas production per ampul. The commercial ampuls, containing 0.33 ml. of isoamyl nitrite and filled under air, were approximately one year old.

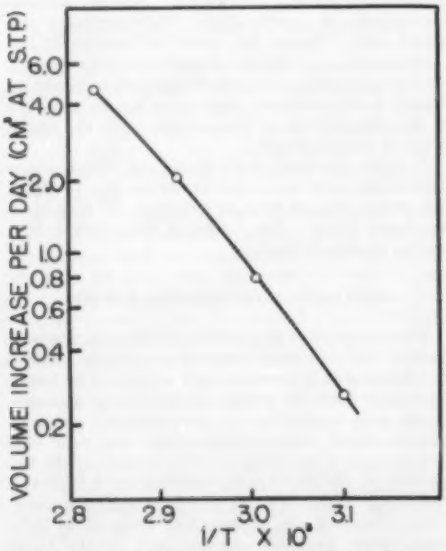

Fig. 2.—An Arrhenius type plot for the decomposition of isoamyl nitrite in commercial ampuls. The ampuls were approximately one year old and contained 0.33 ml. of isoamyl nitrite filled under air.

In considering the rate of decomposition which was found for isoamyl nitrite in sealed ampuls, it becomes apparent that the reaction involved is different from that proposed by Steacie for the thermal decomposition of simple nitrite esters in the vapor phase. Steacie found that the rate of decomposition could be expressed in the form of the following rate equation

$$k = 1.39 \times 10^{14} e^{-37,700/RT}$$

for the overall reaction:

$$2RCH_2ONO \rightarrow RCHO + RCH_2OH + 2NO$$

It can be calculated from this rate equation that a period of twenty-four years would be required to give 10% decomposition at 70°. In the present study, it can be shown that 4.17 ml. of gas represents a minimum of 10% decomposition in ampuls containing 0.5 ml. of isoamyl nitrite, since one mole of the ester will decompose to form $^1/_2$ mole of nitrogen or nitrous oxide. If the initial rate is considered in the plot of gas volume against time for isoamyl nitrite alone (Fig. 3, under air and vacuum), it can be seen that sixteen days is required to produce 4.2 ml. of gas. Considering the final rate, only 2.6 days is necessary to give this amount of gas. This very great difference in rates of decomposition found in the two studies can only be accounted for by a difference in reactions. This is also evident from the considerably lower heat of activation which was found for the degradation of isoamyl nitrite in ampuls.

INFLUENCE OF OXYGEN, WATER, ALCOHOL, AND VOLUME OF ISOAMYL NITRITE PER AMPUL

A second phase of this study included an investigation of the influence of oxygen, water, alcohol, and

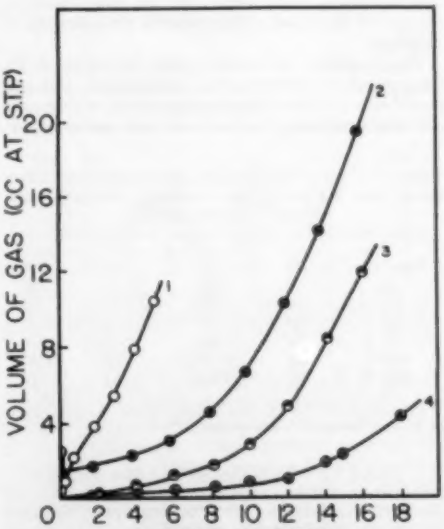

Fig. 3.—A plot showing the effect of oxygen and sodium phosphate (solid) on the rate of gas formation at 70° in ampuls containing 0.5 ml. of isoamyl nitrite. (*1*) Ampuls filled under 1.7 atmospheres of oxygen; (*2*) ampuls filled under air; (*3*) ampuls filled under vacuum; (*4*) ampuls filled under vacuum with 1% w/v trisodium phosphate added.

volume of nitrite ester per ampul on the rate of gas formation. It was felt that this type of information would provide valuable clues concerning the causes of instability of the nitrite ester in ampuls.

Effect of Oxygen.—It was noted previously that analysis of the gases present in ampuls which had been filled under air showed no oxygen present, indicating that oxygen was probably reacting during the breakdown. For this reason, it was felt desirable to investigate the effect of oxygen on the rate of gas formation.

The data from this study are summarized in Fig. 3. When ampuls are filled under vacuum, there is a considerable period during which the formation of gas is relatively slow. After the eighth day at 70°, there is a very rapid increase in the rate, and it finally becomes linear with time. The shape of the curve suggests that autocatalysis is taking place during the reaction. It is highly probable that the first portion of the curve represents the period during which the catalytic species is slowly formed. After the concentration of the species reaches a sufficient level, the catalytic effect becomes very pronounced, accounting for the final rapid rate of gas formation. The linearity of the limiting rate with time might represent a balancing effect between the formation and disappearance of the catalytic species.

The same type of curve was obtained when the ampuls were filled under air. However, the time required to reach a rapid rate of gas formation was considerably less, which suggests that oxygen is capable of forming the catalytic species.

When the ampuls were filled under oxygen, nearly all of the oxygen had disappeared at the end of one hour. Then the rate of gas formation became rapid without showing any induction period. The oxygen evidently caused a sufficient concentration of the catalytic species to be formed immediately, resulting in an initial rapid rate. It is interesting to note that the final slope of the curves for the three cases are quite similar.

Since nitric oxide appears to be an intermediate product of the reaction, oxygen apparently converts this gas to nitrogen dioxide. The nitrogen dioxide may be a catalytic species, or may react further to form another catalytic species. The fact that a catalytic effect was noted even when the ampuls were filled under vacuum indicates that oxygen is not necessary to form the catalytic species.

Effect of Water.—The fact that nitrite esters would be expected to undergo hydrolysis quite readily in the presence of water, especially if acid is present, prompted a study of the effect of water on the reaction. As can be seen from Fig. 4, 0.8% water approximately doubles the rate of gas formation in the early stages of degradation. This does not seem like a very large effect if hydrolysis of the ester were primarily responsible for the breakdown. The fact that the effect is not as large as might be expected if hydrolysis were a factor could possibly be explained by the slight solubility of water in isoamyl nitrite. Also, acid may be present only in very small quantity during the first part of the degradation, and thus, may not appreciably catalyze the hydrolysis.

Effect of Dehydrating Agents.—In view of the effect of water, it was expected that dehydrating agents might decrease the degradation. Figure 4

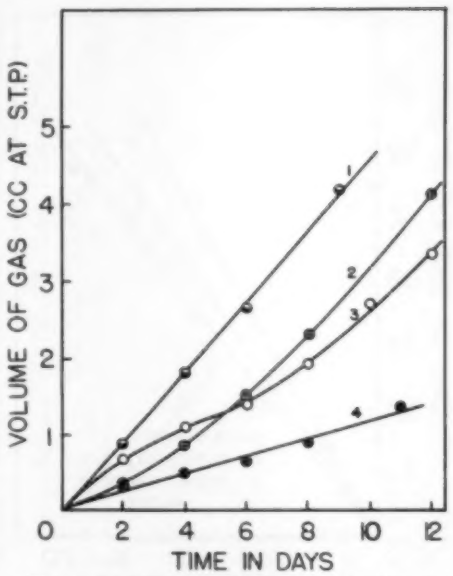

Fig. 4.—A plot showing the effect of water, calcium sulfate, and magnesium sulfate on the rate of gas formation in ampuls containing 0.5 ml. of isoamyl nitrite filled under vacuum and kept at 60°. (*1*) 5% w/v anhydrous magnesium sulfate; (*2*) 0.8% v/v water; (*3*) 10% w/v anhydrous calcium sulfate; (*4*) isoamyl nitrite alone.

shows surprisingly that both anhydrous calcium sulfate and magnesium sulfate increased the rate of gas formation about to the same extent as water. This apparent ambiguity is in all probability caused by some unknown catalytic effect of the dehydrating agents upon the reaction. Possibly, the agents possess sufficient acidity to catalyze the breakdown of the ester.

Effect of Ethanol and Nitrobenzene.—It can be seen from Fig. 5 that alcohol does not have any significant effect on the rate. This experiment was conducted at 51°, and therefore the rate of degradation was relatively slow with no autocatalytic effect noted. Isoamyl nitrite is known to undergo a transesterification reaction with ethyl alcohol to form ethyl nitrite. This reaction could increase the formation of gas only if ethyl nitrite decomposes at a much faster rate than isoamyl nitrite. Since only 1% ethyl alcohol was added, the amount of ethyl nitrite produced would necessarily be small.

Nitrobenzene did not show any significant effect on the rate of gas formation as shown in Fig. 5.

Effect of Volume of Isoamyl Nitrite per Ampul.—When the rates of gas formation at different volumes of isoamyl nitrite from Fig. 6 are plotted against volumes of isoamyl nitrite per ampul, a straight line is obtained (Fig. 7). This shows that the rate of gas production per ampul is directly proportional to the volume of isoamyl nitrite per ampul. Since the total volume of the ampuls was always 2.10 ml., the void space above the liquid decreased as the volume of the liquid was increased. The results indicated that the gas produced by degradation is

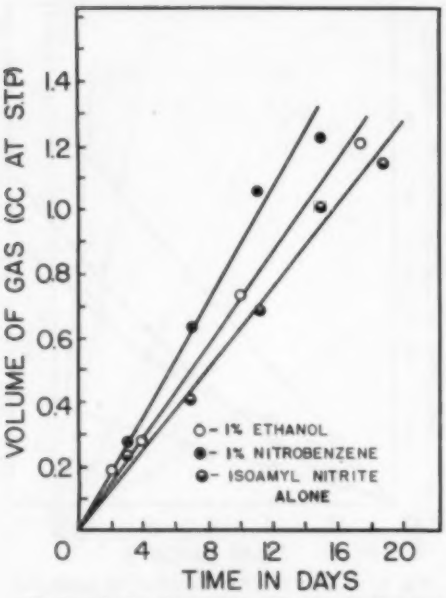

Fig. 5.—A plot showing the effect of nitrobenzene and ethyl alcohol on the rate of gas formation per ampul at 51°. The ampuls were filled under vacuum and contained 0.5 ml. of isoamyl nitrite.

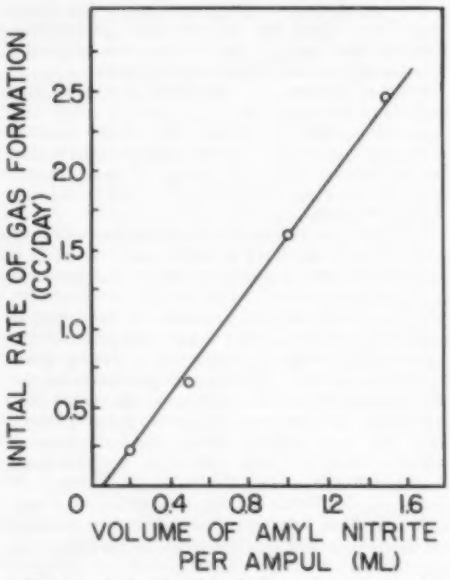

Fig. 7.—A plot showing the rate of gas production per ampul to be directly proportional to the volume of isoamyl nitrite per ampul.

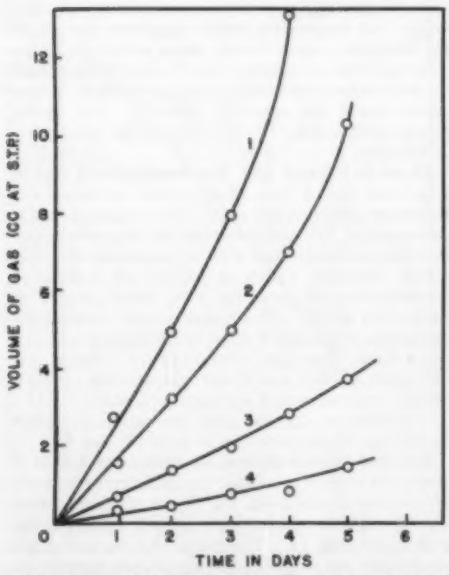

Fig. 6.—A plot showing the effect of varying the volume of isoamyl nitrite per ampul on the rate of gas production at 70°. The ampuls were filled under vacuum. (*1*) 1.5 ml. per ampul; (*2*) 1.0 ml. per ampul; (*3*) 0.5 ml. per ampul; (*4*) 0.2 ml. per ampul.

largely the result of reactions occurring in the liquid phase. This does not necessarily mean that gas phase reactions are absent since the amount of isoamyl nitrite in the gas phase as compared to that in the liquid phase is so small as to make any gas phase reaction appear negligible as far as contribution to the gaseous products is concerned. This study indicates that the practical shelf life of amyl nitrite ampuls is dependent upon the volume of isoamyl nitrite added to the ampuls.

INFLUENCE OF ACID

Since it has been shown by other workers that acid may be a product of the reaction (6), the effect of addition of strong acid to isoamyl nitrite was investigated. Trichloroacetic acid in concentrations varying from 0.16 to 2% was added to ampuls containing 0.5 ml. of the ester.

It can be seen from Fig. 8 that the reaction is very sensitive to acid. When acid is added in concentrations of 0.5 to 2%, the rate of initial gas formation is very rapid, but gradually decreases to a constant value. The nature of the curves suggests that different reactions are responsible for the initial and final stages of degradation. It can be seen from Fig. 9 that the initial rate is proportional to the square of the acid concentration. This suggests that two molecules of acid are involved in a reaction with the nitrite ester. It can be seen from Fig. 10 that the terminal rate is directly proportional to the acid concentration which would indicate that only one molecule of acid is involved in that reaction.

The behavior in the presence of strong acid during the initial phase might be accounted for by the acid catalyzing a transesterification reaction between

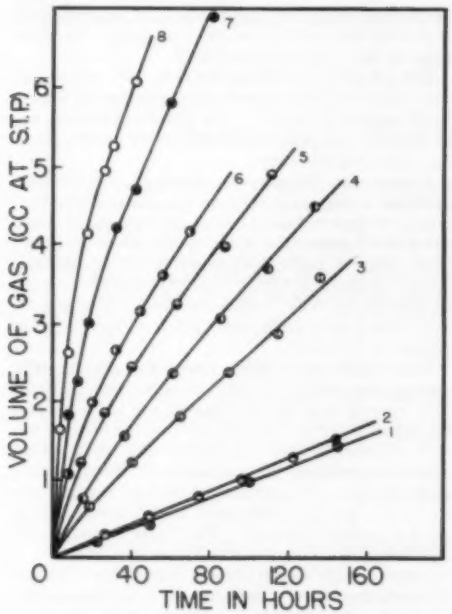

Fig. 8.—A plot showing the effect of varying the concentration of trichloroacetic acid on the rate of gas production in ampuls containing 0.5 ml. of isoamyl nitrite filled under vacuum and kept at 60°. (*1*) Isoamyl nitrite alone; (*2*) 0.17% acid; (*3*) 0.46% acid; (*4*) 0.65% acid; (*5*) 0.87% acid; (*6*) 1.09% acid; (*7*) 1.51% acid; (*8*) 2.03% acid.

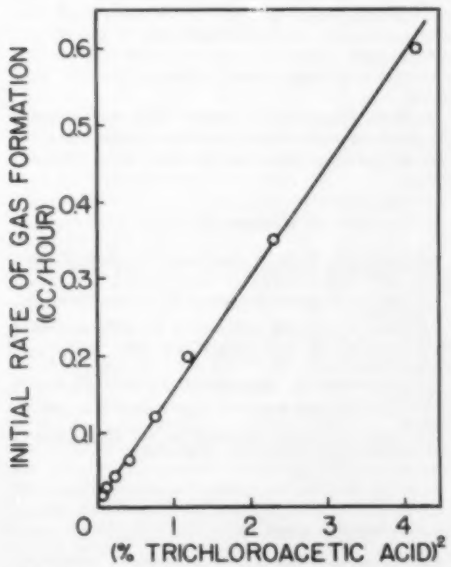

Fig. 9.—A plot showing the initial rate of gas production per ampul to be proportional to the square of the concentration of acid added to isoamyl nitrite.

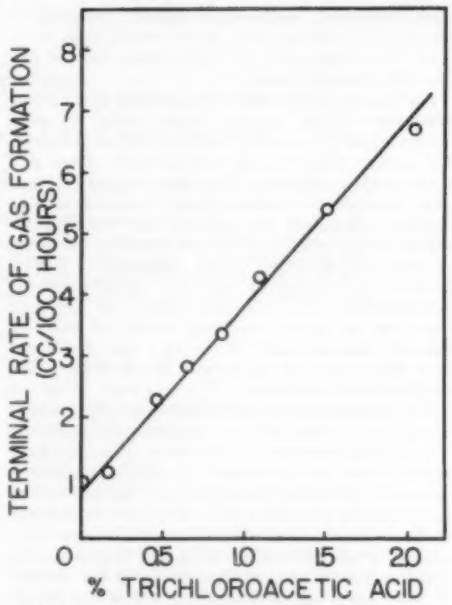

Fig. 10.—A plot showing the terminal rate of gas production per ampul to be directly proportional to the concentration of acid added to isoamyl nitrite.

itself and the nitrite ester. This could be represented as follows:

$$\mathrm{RONO} + \mathrm{CCl_3CO_2H} \xrightarrow{\mathrm{CCl_3CO_2H}} \mathrm{CCl_3CO_2R} + \mathrm{HNO_2}$$

$$2\mathrm{HNO_2} \rightarrow \mathrm{H_2O} + \mathrm{NO_2} + \mathrm{NO}$$

In a previous section, it was shown that the nitric oxide or nitrogen dioxide probably reacts further with aldehyde to produce a mixture of gases containing nitrogen as the principal component.

In view of the fact that water has been shown to be a product, it is evident that trichloroacetic acid can catalyze the hydrolysis of the ester, as shown below;

$$\mathrm{RONO} + \mathrm{H_2O} \xrightarrow{\mathrm{CCl_3CO_2H}} \mathrm{ROH} + \mathrm{HNO_2}$$

$$2\mathrm{HNO_2} \rightarrow \mathrm{NO} + \mathrm{NO_2} + \mathrm{H_2O}$$

The above results show that if a strong acid were formed during the degradation, it would in all probability show a catalytic effect on the breakdown reaction. The importance of eliminating the presence of acid in commercial amyl nitrite is also pointed to by the results of this study.

EXPERIMENTAL

Reagents.—The nitric oxide was prepared by the nitrometer reaction since commercial nitric oxide is often contaminated with small amounts of nitrogen dioxide. The isoamyl nitrite was Fisher reagent grade distilled under vacuum prior to being used. A sample of *n*-butyraldehyde was obtained from a commercial lot by fractional distillation on a fifty

plate Oldershaw fractionating column; analysis of this sample revealed that it was better than 98% pure. The remainder of the chemicals were of the analytical reagent grade.

Experimental Procedure for Analysis of Gaseous Products.—Liquid samples were placed in calibrated ampuls by means of a standardized dropper. The ampul was then placed in a bath of dry ice-acetone and evacuated. The various gases in question were allowed to enter the evacuated ampul through a system of stopcocks and the freezing bath withdrawn in order to allow the ampul to warm up to room temperature. When temperature equilibration was reached the pressure was adjusted to atmospheric. The stopcock connecting the gas source to the ampul was then turned off and the freezing bath replaced. By using a very fine flame from a blowpipe the ampul was sealed off from the remainder of the system.

The gas analysis was performed by gas chromatography using a silica column as previously described.

Gasometric Analytical Procedure.—An analytical method which permitted the volumes of gas produced in ampuls by degradation to be measured proved the most suitable for this study. A schematic drawing of the apparatus is shown in Fig. 11. The method consists essentially of breaking the ampuls in an evacuated chamber and then sweeping the decomposition gases into a semimicro nitrometer (Pregl type) with carbon dioxide. The nitrogen containing decomposition gases displace the 50% potassium hydroxide in the nitrometer whereas the carbon dioxide is completely absorbed by the alkali. The volumes of gas are read in the graduated part of the nitrometer at existing barometric pressure and temperature, and are subsequently converted to volumes at standard conditions. Before breaking each ampul, a small amount of carbon dioxide was passed into the nitrometer to see if the air had been completely swept out of the system. The breaking chamber was then evacuated and the ampul broken by lowering a polyethylene coated iron bar onto it with an electromagnet. The gas was next swept into the nitrometer and the final volume read after the bubbles of gas completely dissolved again in the potassium hydroxide.

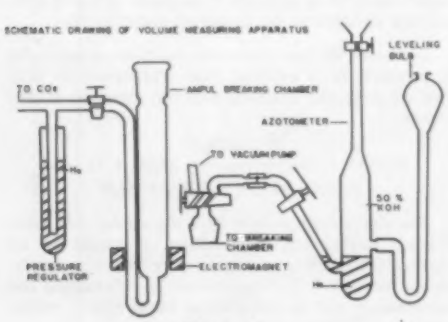

Figure 11.

The solubility of nitrous oxide in 50% potassium hydroxide was determined since this gas is appreciably soluble in water. The gas was found to be, for practical purposes, insoluble under conditions of the analytical procedure.

Ampuls for Gasometric Study.—Commercially available ampuls made of resistant glass, and having a total volume of approximately 2 ml. were used. In all of the experiments, except for when the effect of changing the volume of isoamyl nitrite was studied, 0.5 ml. of isoamyl nitrite was introduced into each ampul with a 1.0-ml. volumetric pipet. The ampuls were sealed at a calibration mark to have a total volume of 2.10 ml.

The ampuls were filled under vacuum by connecting the ampul directly to a vacuum system. The ampuls containing isoamyl nitrite were immersed in a dry ice–acetone mixture during evacuation to prevent loss of the ester and then sealed while remaining in the mixture. In filling ampuls under oxygen, the gas was allowed to displace the air in the ampuls while they were immersed in a dry ice–acetone mixture. They were subsequently sealed.

Commercially made ampuls containing 0.33 ml. of amyl nitrite were used for the temperature dependency study. These ampuls were approximately one year old. Ampuls were taken from a single commercial lot for each temperature studied. The volumes of gas from at least four ampuls were averaged for the final reading at each time interval. In all of the other experiments, the volumes from two ampuls were averaged for each reading.

Decomposition of Samples in Gasometric Study.—Each batch of ampuls was placed in a light resistant, wide mouth bottle, and the bottle was placed in a thermostatically controlled bath set to the desired temperature. Samples were withdrawn at suitable intervals and were stored in the refrigerator until used.

A blank consisting of ampuls filled with isoamyl nitrite alone was run for each experiment since the rate of gas formation was found to differ between blanks.

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Kinetics of Degradation of Amyl Nitrite Ampuls II.*

Stabilization

By MARTIN H. YUNKER† and TAKERU HIGUCHI

It is shown that the stability of amyl nitrite ampuls can be greatly increased by the addition of various substances—including potassium carbonate (solid), trisodium phosphate (solid), magnesium oxide (solid), pyridine (dissolved), and diphenylamine (dissolved). The mechanism of stabilization appears to involve behavior of these agents as sinks for nitrogen dioxide and/or nitric acid produced during the breakdown. The degree of stabilization achieved suggests that the shelf life of the nitrite ester can be readily prolonged as much as tenfold as compared to present commercial products.

SINCE isoamyl nitrite (amyl nitrite U. S. P.) undergoes serious degradation in ampuls, with formation of considerable gas, the present study was undertaken to investigate possible methods of stabilization. No previous attempt appears to have been made to stabilize alkyl nitrites.

In a previous publication (1), it was suggested that the relatively rapid formation of gas in amyl nitrite ampuls was due to the autocatalytic nature of the reaction, a catalytic species being progressively produced during the degradation process. Since it was shown that the rate was substantially increased by addition of a strong acid, the possibility arose that an acid might be accelerating the decomposition. Investigations were, therefore, conducted to determine the effect of alkaline substances on the rate of gas formation in amyl nitrite ampuls.

EXPERIMENTAL

Reagents.—The isoamyl nitrite used for this study was reagent grade material which was vacuum distilled immediately before use, the middle fraction from the distillate being employed.

Procedure.—The experimental procedure and the method of analysis were essentially the same as those used for Part I of this series.

The agents used in this study fell into four categories: (A) a strong base (triethylamine); (B) solid acid absorbents (potassium carbonate, sodium phosphate, magnesium oxide, and sodium bicarbonate); (C) a weak base (pyridine); and (D) those amines which are neutral, but might be expected to react with nitric acid (diphenylamine, *para*-nitromethylaniline). The effects of the agents were studied in 1 to 2% w/v concentration added to ampuls containing 0.5 ml. of isoamyl nitrite. Generally the ampuls were filled under vacuum unless otherwise indicated. The water-soluble salts were added to isoamyl nitrite by dissolving them in a measured volume of distilled water, and then introducing a suitable aliquot of this solution into the ampul. The water was subsequently evaporated off, and the isoamyl nitrite then added. A blank consisting of ampuls filled with isoamyl nitrite alone was run for each experiment.

Analysis of the Carbonate.—The residue from several ampuls of isoamyl nitrite to which 5% carbonate had been added was separated by filtration, and washed thoroughly with ether to extract all of the isoamyl nitrite. Qualitative tests were used to determine if nitrite and nitrate were present. Total nitrite and nitrate were determined quantitatively by the nitrometer method (2), and nitrite alone by the permanganate method (3).

RESULTS

Strong Base.—The effect of the addition of 1% v/v triethylamine to isoamyl nitrite on the rate of gas formation per ampul is shown in Fig. 1. It is seen that the rate is very rapid at first, and then decreases as the reaction proceeds. Since the rate is considerably greater than with isoamyl nitrite alone, it appears that strong bases are not suitable for stabilization of isoamyl nitrite.

After addition of the triethylamine to isoamyl nitrite, a precipitate formed immediately, indicating that a reaction had taken place. Possibly the nitrite salt of the tertiary amine was formed by cleavage of the ester, in some manner.

Solid Acid Absorbents.—The data in Fig. 2 show that when 2% w/v potassium carbonate is added to the isoamyl nitrite ampuls, the increase in volume of gas per ampul is much less than that for isoamyl nitrite alone. These results indicate that isoamyl nitrite can be stabilized in ampuls by the addition of alkaline salts which are capable of reacting with an acidic, catalytic specie formed during the degradation. It might be expected that the ability to neutralize the acid, and thus stabilize the ester, would depend upon the amount of the alkaline salt added. This is shown to be true by the data in Fig. 3, from which it can be seen that 0.1% carbonate was very slightly effective whereas 2% carbonate was very effective in stabilizing the isoamyl nitrite. As the concentration of the carbonate was increased, the induction period became longer, and finally with 2% carbonate, no catalytic effect appeared in the curve.

If a 10 degree temperature coefficient of 2.4 is used for the reaction (1), the 19 day period of stabilization with 2% carbonate shown in Fig. 3 is equivalent

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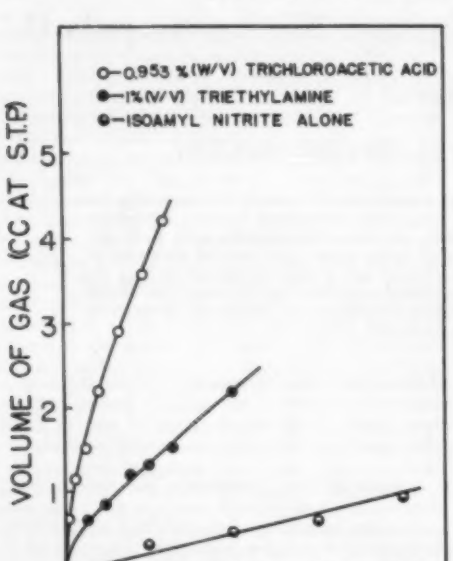

Fig. 1.—A plot showing the effect of triethylamine and trichloroacetic acid on the rate of gas formation per ampul at 60°. The ampuls contained 0.5 ml. of amyl nitrite filled under vacuum.

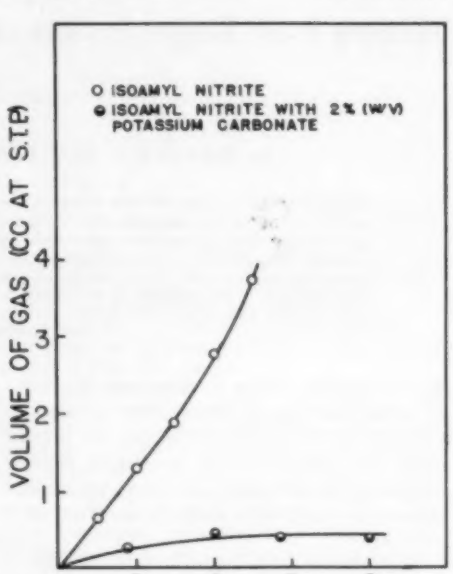

Fig. 2.—A plot showing how the addition of potassium carbonate (solid) to amyl nitrite affects the rate of gas production per ampul at 70°. The ampuls contained 0.5 ml. of amyl nitrite filled under vacuum.

to 2 years at room temperature. The volume of gas (1.46 cc.) produced during this period is much less than that amount (7 to 8 cc.) found necessary to cause commercial ampuls to explode. It should be noted also that commercial ampuls contain only 0.33 ml. of isoamyl nitrite, thus the volume of gas produced in them would be less than in this experiment where the ampuls contained 0.5 ml. It was shown in the previous article that the rate of gas production was directly proportional to the volume of isoamyl nitrite in the ampuls.

Sodium phosphate was also found to stabilize isoamyl nitrite in ampuls against gas formation, but to a slightly lesser degree than carbonate (Fig. 4). Magnesium oxide was more effective than sodium phosphate, perhaps because it is more alkaline. It can be seen in Fig. 5 that 2% w/v sodium bicarbonate substantially reduces the volume of gas per ampul. However, it is the least effective of the above acid absorbents, probably because it is also the least alkaline.

Since the above studies were conducted at 70°, it was decided to test the effectiveness of alkaline salts at a lower temperature. Four batches of ampuls containing: isoamyl nitrite filled under vacuum, isoamyl nitrite with 2% Na_3PO_4 filled under vacuum, isoamyl nitrite with 2% Na_3PO_4 filled under air, and isoamyl nitrite with 2% K_2CO_3 filled under vacuum were heated at 50° for 12 weeks. The results are summarized in Table I. They show that potassium carbonate is more effective at a lower temperature, having completely stopped the production of gas during a 12-week period. Sodium phosphate is seen to have substantially reduced the

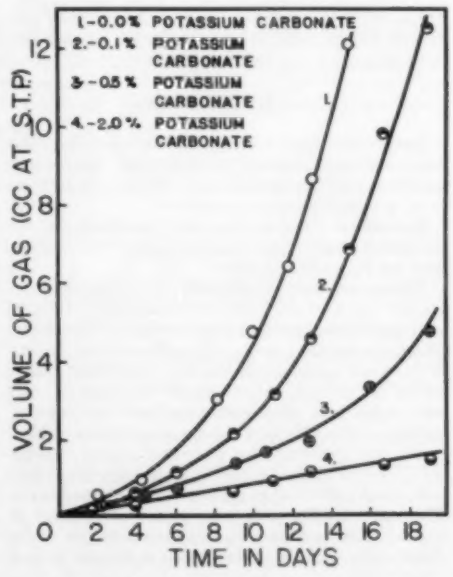

Fig. 3.—A plot showing the effect of varying the concentration of potassium carbonate on the rate of gas production per ampul of amyl nitrite at 70°. The ampuls contained 0.5 ml. of amyl nitrite filled under vacuum.

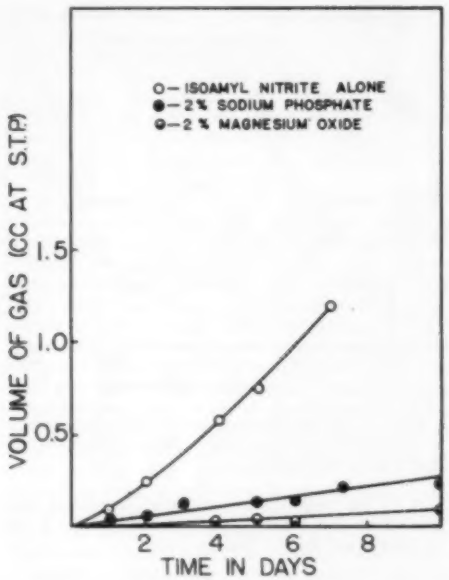

Fig. 4.—A plot showing the effect of trisodium phosphate (solid) and magnesium oxide (solid) on the rate of gas formation in ampuls containing 0.5 ml. of amyl nitrite filled under vacuum.

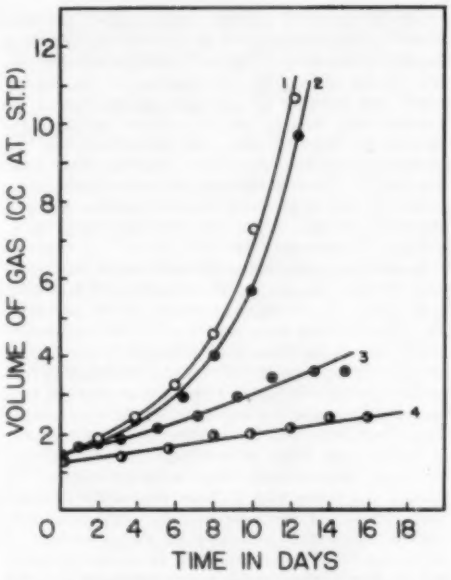

Fig. 5.—A plot showing how the addition of *p*-nitromethyl aniline (dissolved), pyridine (dissolved), and sodium bicarbonate (solid) affects the rate of gas production per ampul at 70°. All ampuls contained 0.5 ml. of amyl nitrite filled under air. *1*, 1% *p*-nitromethyl aniline; *2*, isoamyl nitrite alone; *3*, 2% sodium bicarbonate; *4*, 2% pyridine.

amount of gas, both in ampuls filled under vacuum and under air.

The residue from ampuls stabilized with potassium carbonate responded positive to qualitative tests for nitrite and nitrate. Results of a quantitative analysis of the residue for nitrite and nitrate showed that 2.3 moles of nitrate were formed for each mole of nitrite, and that 40% of the 25 mg. of carbonate in each ampul had been neutralized. Since one mole of isoamyl nitrite will react to form one mole of nitrite or of nitrate, it can be calculated that 3.8% of the ester had been decomposed by the above route. In considering that the experiment was run at 80° for 14 days, this is a relatively small amount of degradation. The nitrate found by the analysis could possibly be accounted for by a reaction between nitrogen dioxide and carbonate as reported by Addison and Lewis (4). This would be represented by the following equation:

$$K_2CO_3 + 3NO_2 \rightarrow 2KNO_2 + CO_2 + NO$$

However, nitrate might also be formed by neutralization of nitric acid. The nitrite is most probably formed by neutralization of nitrous acid by the carbonate. These results definitely suggest that nitric acid or nitrogen dioxide is the catalytic specie formed during the degradation.

Pyridine.—It was desirable to determine what effect a weak organic base might have on the reaction since it was previously shown that a strong organic base increased the degradation. It can be seen from Fig. 5 that the addition of 2% v/v pyridine to amyl nitrite ampuls greatly decreases the volume of gas formed after a considerable period of storage at 70°. As with potassium carbonate, the pyridine has completely eliminated the autocatalysis

TABLE I.—THE VOLUMES[a] OF GAS FORMED AT 50° IN AMPULS CONTAINING 0.5 ML. OF AMYL NITRITE AFTER ADDITION OF 2% (W/V) OF K_2CO_3 AND Na_3PO_4

Storage Time	0 wk.	2 wk.	4 wk.	6 wk.	8 wk.	10 wk.	12 wk.
Amyl Nitrite—under vacuum	0	1.18	2.98	4.35	11.6	All ampuls had broken	
2% Na_3PO_4 added—under vacuum	0	0.57	0.98	1.10	1.57	1.82	2.78
2% Na_3PO_4 added—under air	1.24	1.39	1.62	1.78	2.14	2.42	2.75
2% K_2CO_3 added—under vacuum	0	0.09	0.09	0.15	0.19	0.16	0.04

[a] Volumes of gas in ml. corresponding to 0° at 760 mm. of mercury pressure.

noted for isoamyl nitrite alone. Since pyridine would not be expected to be nitrated by nitrogen dioxide, it appears that it may stabilize the ester by neutralizing nitric acid. In support of this theory, Addy and Mcbeth (5), attempting nitrosation of pyridine with isoamyl nitrite, isolated pyridine nitrate from a mixture of the two which had been allowed to stand for a few days. The fact that pyridine exhibits a favorable influence whereas triethylamine did not is probably due to pyridine being a much weaker base, and thus, not being able to cause cleavage of the ester.

Diphenylamine.—Since diphenylamine has been used for the stabilization of nitrocellulose, it seemed advisable to investigate its effect on the reaction. From Fig. 6 it can been seen that 1% w/v diphenylamine essentially stops the formation of gas during 14 days of storage at 70°. Upon addition of the amine, the color of isoamyl nitrite was changed to a deeper yellow, and a precipitate formed after several days of heating. The yellow precipitate melted over a considerable range of temperature, and did not show any improvement after recrystallization. It gave a red color with sodium hydroxide solution characteristic of nitro compounds. The solid was chromatogrammed according to the procedure of Schroeder (6). After repeated rechromatography, two colored compounds were separated on the column which were identified as 2,4′-dinitrodiphenylamine and 4,4′-dinitrodiphenylamine by comparing their ultraviolet spectra (Fig. 7) with those listed for dinitro derivatives of diphenylamine by Schroeder (7). The location of the maxima and minima agree very well; however, the ratios of D max./D

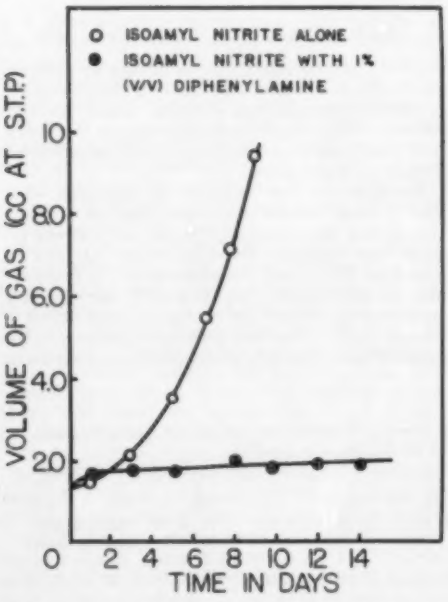

Fig. 6.—A plot showing how the addition of diphenylamine (dissolved) to amyl nitrite affects the rate of gas production per ampul at 70°. The ampuls contained 0.5 ml. of amyl nitrite filled under air.

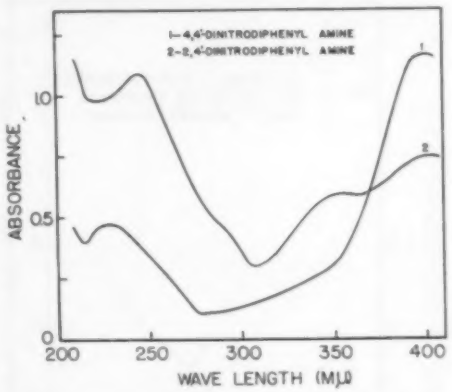

Fig. 7.—Ultraviolet spectra of the nitro derivatives of diphenylamine formed when diphenylamine is added to amyl nitrite.

min. of our compounds were lower, indicating that they were less pure. The melting points of our compounds were also slightly lower:

	Listed	Found
2,4′-dinitro D. P. A.	220°	217°
4,4′-dinitro D. P. A.	216°	210°

The color reactions were identical to those reported by Ashdown and Davis (8).

These results indicate that diphenylamine is probably nitrated by nitric acid formed during the reaction. It is interesting to note that Ashdown and Davis were able to isolate the same two dinitro derivatives from nitrocellulose which had been stabilized with diphenylamine. Schroeder reports that diphenylamine stabilizes nitrocellulose by continuously combining with nitrogen acids and oxides which arise during the decomposition, and thus, decreases the autocatalytic effect of these products on the further decomposition of the nitrocellulose. There are two possible mechanisms by which the dinitro derivatives might be formed: (A) the classical method which consists of a series of nitrosations, rearrangements, and oxidations, or (B) a series of nitrosations, denitrosations, and nitrations as proposed by Schroeder (6). The second scheme does not involve any rearrangements or formation of C-nitroso compounds, but only nitrosation of the diphenylamine itself. Although we did not isolate the N-nitroso compound, it is probably formed during the reaction since alkyl nitrites are known to be nitrosating agents.

***Para*-Nitromethylaniline.**—Gallaghan and Pincus (9) reported that N-alkyl nitro anilines which contain an alkyl group such as Me, Et, iso-Pr, and also contain a nitro in either the 3 or 4 position on the ring were superior to diphenylamine for stabilizing nitrocellulose. They stated that the compounds were sufficiently neutral and absorb nitric acid and highly oxidized nitrogen atoms. Therefore, it was thought that these compounds might also prove useful for stabilizing isoamyl nitrite.

The results in Fig. 5 surprisingly show that 1% w/v of *p*-nitromethylaniline increased the volume of gas produced per ampul. Why this compound should not stabilize isoamyl nitrite while diphenyla-

mine does is not easily explained. Possibly the two compounds react with nitric acid by different mechanisms and this in some way accounts for their different behavior toward isoamyl nitrite. It is noteworthy that Gallaghan and Pincus stated that diphenylamine reacts with nitric acid and nitrogen dioxide to produce nitric oxide whereas the N-alkyl nitro anilines rapidly absorb nitrogen dioxide and nitric acid without reducing them.

Confirmation of Stabilization by Infrared Spectral Analysis.—Although it has been shown that carbonate will stop the gas formation in amyl nitrite ampuls, the question existed whether any changes were occurring in the liquid phase. For this reason, the infrared absorption spectrum of isoamyl nitrite which had been heated for 12 weeks at 50° in the presence of carbonate, and had shown no gas formation, and the spectrum of isoamyl nitrite alone that had been heated for 8 weeks at 50° were obtained. In comparing these with the spectrum of pure isoamyl nitrite, it was found that no significant change had occurred in the spectrum of the stabilized isoamyl nitrite. The spectrum of the unstabilized control sample, as expected, indicated that it had undergone extensive degradation, with considerable ester and nitrate formation. These results show that when isoamyl nitrite is stabilized against gas formation in ampuls, it does not undergo any significant decomposition.

DISCUSSION

The results of these studies indicate that the addition of solid acid absorbents such as potassium carbonate, or of diphenylamine, to isoamyl nitrite may prove useful in preventing loss of amyl nitrite ampuls due to gas formation. This would be of particular advantage when the ampuls are stored for a prolonged period.

It has been shown that nitric acid which is formed during the decomposition probably catalyzes the degradation of isoamyl nitrite in ampuls. In the previous article, it was noted that nitric oxide was formed during the reaction, and also that oxygen had a catalytic effect on the degradation. Water was also reported to be a product of the reaction. In considering these facts, the formation of nitric acid might be explained by the following scheme:

$$2NO + O_2 \rightarrow 2NO_2$$
$$3NO_2 + H_2O \rightarrow 2HNO_3 + NO$$

This could account for the catalytic effect of oxygen previously reported. However, since catalysis was also noted when the ampuls were filled under vacuum, it appears that another reaction is involved by which nitric acid can be formed. It was shown previously that all of the oxygen disappeared in a short time from the ampuls, so the above scheme could account for only an initial formation of nitric acid. Further work on the decomposition needs to be done to clarify this phase. The effect of nitric acid on the reaction is probably similar to that of trichloroacetic acid previously noted. It can catalyze the hydrolysis of isoamyl nitrite to the alcohol and nitrous acid with the latter breaking down further to nitric oxide and nitrogen dioxide. There is also some evidence that the acid might be catalyzing a transesterification reaction between itself and isoamyl nitrite,

$$RONO + HNO_3 \rightarrow RONO_2 + HNO_2$$
$$2HNO_2 \rightarrow H_2O + NO + NO_2$$

since it has been shown in the previous article that an organic nitrate is continuously formed during the reaction.

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The Reserpine, Rescinnamine, and Deserpidine Content of Rauwolfia Roots*

By DANIEL BANES, ALBERT E. H. HOUK, and JACOB WOLFF

The alkaloids reserpine, rescinnamine, and deserpidine occurring in the roots of various Rauwolfia species are separated by column chromatography, and determined by ultraviolet spectrophotometry.

SEVERAL METHODS have been published on the determination of reserpine in rauwolfia root. Kidd and Scott (1) isolated the alkaloid by countercurrent distribution, and analyzed the fractions by ultraviolet spectrophotometry. Their assay values on small representative samples from 5-Kg. batches of powdered root were in excellent agreement with the actual yield of crystalline reserpine from the entire batch. Sakal and Merrill (2) employed paper ionophoresis for the separation of rauwolfia alkaloids, and corrected the observed ultraviolet absorption of the reserpine fraction for absorbing contaminants. Dechene (3) extracted the feebly basic alkaloids of *Rauwolfia serpentina* from dilute sulfuric acid

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solution with chloroform, treated the extracted alkaloids with hydrogen peroxide in acid solution, and calculated the fluorescing substances indiscriminately as reserpine.

Carol and his collaborators (4) have analyzed *Rauwolfia serpentina* for both reserpine and rescinnamine. They separated a feebly basic alkaloidal fraction by liquid-liquid partition chromatography on columns, hydrolyzed the alkamine esters, extracted the acids obtained, and determined trimethoxybenzoic and trimethoxycinnamic acids simultaneously by ultraviolet spectrophotometry. The same group (5) later published a colorimetric procedure for the "reserpine-rescinnamine group alkaloids" in *Rauwolfia serpentina*, based on the nitrite test of Szalkowski and Mader (6), and showed that the two methods yielded concordant results. The nitrite procedure was studied collaboratively in several laboratories and is being considered for adoption as the official assay procedure for the National Formulary (7).

Deserpidine, an alkaloid first found in *Rauwolfia canescens* (8), has been stated (9) to occur as a minor constituent in several Rauwolfia species. However, no quantitative data have been published on the concentration of this potent alkaloid in the roots of any species. Hayden, Ford, and Houk (10) have employed column partition chromatography for the complete separation of deserpidine, reserpine, and rescinnamine in eluates suitable for spectrophotometric measurements. We have adapted their procedure to extracts of *Rauwolfia serpentina*, *Rauwolfia canescens*, *Rauwolfia vomitoria*, and *Rauwolfia heterophylla*.

EXPERIMENTAL

Finely powdered rauwolfia root (5–10 Gm.) was extracted in a Soxhlet extractor with alcohol for four hours, and the extract was diluted to 100 ml. The reserpine-rescinnamine group alkaloids in a 20-ml. aliquot were separated by the procedure of Banes, *et al.* (5). The combined chloroform extracts were mixed with 30 ml. of alcohol, and evaporated to dryness under vacuum at a temperature below 70°. The residue was dissolved in about 1 ml. of chloroform and the solution was transferred with the aid of several 0.5-ml. portions of chloroform to a beaker containing 2 Gm. of siliceous earth. After thorough mixing, the solvent was evaporated under vacuum at room temperature, and the residue was incorporated in a chromatographic column, as described by Hayden, *et al.* (10). Consecutive 10-ml. cuts were examined to locate the fractions desired. Those fractions containing high concentrations of deserpidine, reserpine, or rescinnamine were combined and diluted, if necessary, to facilitate spectrophotometric measurements.

Powdered extracts of roots were dissolved in chloroform, and suitable aliquots were mixed with siliceous earth, vacuum-dried and analyzed chromatographically. Tablet samples were powdered, and a quantity equivalent to 5 Gm. of root was analyzed according to the procedure for powdered whole root.

DISCUSSION

The elution pattern of deserpidine, reserpine, and rescinnamine from rauwolfia root extracts was consistent with the observations of Hayden and her collaborators (10). The forerun, containing small quantities of substances with the ultraviolet absorption spectra of 3-dehydroreserpine, ajmalicine, and reserpinine, was usually 100–120 ml. Deserpidine appeared within the next 90 ml. of eluate, reserpine in the next 150–200 ml. cut, and rescinnamine in the following 300 ml. These volumes varied with the concentrations of the individual alkaloids. The ultraviolet absorption of the fractions intervening between the chief alkaloids showed a slight background absorption, equivalent to less than 2% of the reserpine or rescinnamine content in *Rauwolfia serpentina*. Deserpidine occurs in this species in much smaller proportions than the other alkaloids, and the background absorption is accordingly of greater significance in its determination.

The data in Table I on the chromatographic analysis of various rauwolfia preparations show that deserpidine constitutes less than 3% of the total of reserpine, rescinnamine, and deserpidine in the ten authentic samples of *Rauwolfia serpentina* root analyzed. Reserpine concentrations in the whole root varied from 0.10 to 0.16%, with the rescinnamine content about 0.4 those values. In *Rauwolfia canescens* root the average concentration of both deserpidine and reserpine was approximately 0.07%, and rescinnamine was absent. No rescinnamine, and only small proportions of deserpidine were found in *Rauwolfia heterophylla*, while *Rauwolfia vomitoria* contained relatively large quantities of both rescinnamine and reserpine. These findings are in accord with the observations of earlier investigators.

Table II compares the total reserpine plus rescinnamine values obtained by the present method for *Rauwolfia serpentina* roots with reserpine-rescinnamine group alkaloids assays by the nitrite colorimetric procedure. The two sets of values for the powdered roots show an excellent correlation, with the nitrite values greater by a factor of 1.11–1.18. These figures demonstrate that the nitrite assays for reserpine-rescinnamine content of *Rauwolfia serpentina* roots, include a small quantity of other chromogenic alkaloids. The tablet analyses showed greater variation between the two methods. However, both methods indicated correctly the composition of Sample 11, an uncoated tablet fabricated to contain 88 mg. of Sample 4 (*Rauwolfia serpentina* powdered root) and 14 mg. of excipients per tablet. Moreover, both methods agreed that Sample 15 contained slightly more of the active alkaloids than the known Sample 11; that Samples 12 and 13 were weakest and equivalent to each other; and that Sample 14 was intermediate in strength.

Although the present method yielded analytical data for reserpine and rescinnamine in several root samples almost identical with the results obtained by the earlier indirect hydrolytic procedure (4),

TABLE I.—CHROMATOGRAPHIC ANALYSES OF RAUWOLFIA PREPARATIONS

Sample	Description	Mg. per 100 Gm. or per 1,000 Tablets— Deserpidine	Reserpine	Rescinnamine
1	*R. Serpentina* powdered root	5	158	57
2	*R. Serpentina* powdered root	6	143	62
3	*R. Serpentina* powdered root	3	141	66
4	*R. Serpentina* powdered root	4	131	67
5	*R. Serpentina* powdered root	5	128	56
6	*R. Serpentina* powdered root	5	107	48
7	*R. Serpentina* powdered root	5	115	44
8	*R. Serpentina* powdered root	4	103	41
9	*R. Serpentina* powdered root	4	98	45
10	*R. Serpentina* powdered root	4	97	35
11	*R. Serpentina* Tablets 88 mg.[a]	3	119	60
12	*R. Serpentina* Tablets 100 mg.	6	80	23
13	*R. Serpentina* Tablets 100 mg.	5	76	28
14	*R. Serpentina* Tablets 100 mg.	4	90	27
15	*R. Serpentina* Tablets 100 mg.	4	124	64
16	*R. Serpentina* powdered extract[b]	910	33,500	20,000
17	*R. Serpentina* powdered extract[b]	1,100	33,400	17,900
18	*R. Serpentina* powdered extract	32	823	364
19	*R. Serpentina* powdered extract	24	268	168
20	*R. Canescens* powdered root	40	65	0
21	*R. Canescens* powdered root	82	70	0
22	*R. Canescens* powdered root	66	86	0
23	*R. Heterophylla* powdered root	5	87	0
24	*R. Heterophylla* powdered root	2	27	0
25	*R. Vomitoria* powdered root	5	201	41
26	*R. Vomitoria* powdered root	4	127	79

[a] Fabricated to contain 88 mg. of Sample 4 (*R. serpentina* powdered root) and 14 mg. excipients.
[b] Alleged to contain 30–35% reserpine.

TABLE II.—ANALYSES OF *R. serpentina* PREPARATIONS

Sample[a]	Chromatographic Reserpine and Rescinnamine, mg./100 Gm.	Reserpine-Rescinnamine Group Alkaloids (5), mg./100 Gm.
1	215	243
2	205	233
3	207	232
4	198	227
5	184	204
6	155	179
7	159	177
8	144	166
9	143	162
10	132	156
11	179	201
12	103	138
13	104	137
14	117	158
15	188	219

[a] Sample numbers correspond to those in Table I.

some discrepancies were encountered. In the case of one root, rescinnamine and reserpine were 19 and 12% higher, respectively, by the indirect method than by the chromatographic method. The present procedure is probably more reliable, since it isolates the alkaloids sought, and determines them individually by direct measurements.

SUMMARY

A procedure for the chromatographic separation of reserpine, rescinnamine, and deserpidine has been utilized for the determination of these alkaloids in various Rauwolfia species. Analytical data for *Rauwolfia serpentina* powdered root show excellent correlation with assay values for reserpine-rescinnamine group alkaloids obtained by the nitrite colorimetric procedure.

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The Uniformity of Distribution of Phosphorus Compounds in Tablet Matrices Using Radioactive Tracer Techniques*

By GILBERT S. BANKER, JOHN E. CHRISTIAN, and H. GEORGE DeKAY

Radioactive tracer techniques and statistical methods have been applied to study the uniformity of distribution of several additive phosphorus-32 labeled compounds in a lactose excipient in tablet matrices. Certain physical properties of the additive and excipient powders, the duration of mixing, methods of granulation and drying, solubility of the additive in the granulating agent, additive migration, and other factors, were studied for their effect on the uniformity of additive distribution.

UNIFORMITY and accuracy of drug dosage is considered an essential requirement of most pharmaceutical dosage forms. One of the three major objections which have been made against tablets as a dosage form has been the possibility of nonuniformity of dosage (1, 2). For perhaps this reason Burlinson (3) lists accurate dosage as the most important requirement of a good tablet. Accurate dosage in tablets is controlled by constant tablet weight and a uniform distribution of the additive material throughout the tablet granulation.

With the advent of pharmaceutical drug products of greater potency it is no longer safe to rely on tablet weight variation as the criterion for the uniformity of medicament dosage in tablets. It is obvious that a uniform distribution of potent drugs is necessary in tablet matrices since slight variation in uniformity could produce tablets with more than a slight variation in dosage, particularly when the additional nonuniformity effect of weight variation is considered.

It is essential that the uniformity of drug dispersion in the tablet excipient and the factors affecting this uniformity be known and understood, especially in the preparation of tablets containing only a few milligrams of active ingredient. Existing reports of the factors affecting the uniformity of tablet dosage are few in number and often hypothetical since they completely lack the substantiation of laboratory experimentation.

EXPERIMENTAL

Bloom and Livesey (4) in their distribution studies of additives in animal feeds describe the use of a radioactive isotope as the method of analysis. Necessary modifications of their procedure were adopted using phosphorus-32 as an isotope possessing desirable properties for studying additive distribution in tablet matrices. Phosphorus-labeled monobasic potassium phosphate was selected as a water-soluble additive and phosphorus-labeled phosphoric acid was selected as a water and alcohol-soluble additive. The salt was prepared by reaction of $H_3P(P^{32})O_4$ with anhydrous potassium carbonate, analytical reagent.

A lactose excipient was prepared by drying lactose U. S. P. at 60°, prescreening the dried powder through a number 30 sieve to remove lumps, and finally separating the powder into particle size sieve fractions by a sieve method. Five 5-inch diameter American Society of Testing Materials standard sieves were placed from coarse to fine in a motor driven shaker to separate 100-Gm. portions per separation into sieve fractions. The lactose sieve fractions were analyzed for certain of their properties, including true density and apparent density in order to determine void volume and its significance on uniformity of additive distribution.

Powder mixtures of 0.50% one hundred-mesh labeled phosphate salt in lactose were used in this and subsequent experiments. The specific activity of each phosphorus-labeled monobasic potassium phosphate sample was calculated. Sufficient monobasic potassium phosphate carrier was added to the labeled salt so that the initial corrected activity of a 10.0-mg. sample of lactose powder mixture, containing 0.5% labeled material, would be between 500 and 1,000 counts per minute. Powder mixing was accomplished with a Rampe tumbler mixer,[1] using powder loads of 30 to 1,500 Gm. and mixing jars with a volume greater than three times that of the powder being mixed. Ten or more 10.0-mg. samples were taken from each powder mixture, granulation, or tablet to be studied, weighed on a microbalance, dissolved in water on individual aluminum counting cups, oven dried at 50°, and counted for activity. Each sample activity was corrected for background and resolving time according to the U. S. P. XV method (5). For the measurement of radioactivity a mica window, 2.4 mg./cm.^{2} Gieger-Mueller tube[2] was used. The uniformity of activity of the ten or more samples was determined by calculating the per cent coefficient of variation in each instance.

Autoradiographs of granulations and tablets were prepared to provide a gross visual means of

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[1] Rampe Manufacturing Co., Cleveland, Ohio.
[2] Tracerlab, Inc., 130 High Street, Boston 10, Mass.

studying the distribution of radioactive additive and to verify the method of random sampling and statistical analysis of sample activities for uniformity.

RESULTS AND DISCUSSION

Uniformity Studies of Powder Mixtures.—Mixing studies were conducted by mixing combinations of particle size sieve fractions of additive and excipient for different periods as shown in Table I. Uniformity of distribution of the additive (labeled phosphate) in the powders, granulations, and tablets is reported in the following tables as $V\%$. This is simply the standard deviation/the mean, the expression multiplied by 100 for expression as a per cent. Thus a high $V\%$ means poor distribution of phosphate and a low $V\%$ means a more uniform distribution.

When the particle size of labeled phosphate salt and lactose was simultaneously varied (Table I, powder mixtures 1 through 4), the 200-mesh powder mixture was found to be uniform (powder number 4) and the coarser powder fractions were significantly less uniform (powders number 1, 2, and 3). When the four different particle size fractions of labeled salt were mixed three, fifteen, and thirty minutes with separate 200-mesh portions of excipient powder, the duration of mixing did not significantly affect the uniformity of the coarser three sieve separated fractions (compare powder mixtures 5 through 7, 8–10, and 11–13), but did significantly influence the uniformity of the 200-mesh powders (compare powder mixtures 14–16). The finer particle size distributions of additive require a longer duration of mixing to achieve their optimum uniformity than did the coarser particle size distributions. A fifteen minute tumble mixing was adequate for the 200-mesh powders. For maximum uniformity of additive dispersion in this particular powder mixture of additive and excipient, the results of Table I indicate a 150/200 or 200-mesh additive should be used with a 200-mesh excipient powder.

The void volume (8) of lactose excipient in powders Nos. 17–19 (Table I) was found to be 0.72, 0.84, and 0.86 ml./Gm. The coefficient of variation of these powder mixtures was 59.5, 36.2, and 17.3%. Thus in this case, the differences in distribution between 17 and 19 and 18 and 19 are not due to differences in void volume, but in mesh size of the lactose.

Uniformity Studies of Granulations.—The relative solubility of the granulating agent for the additive and the amount of granulating agent used were studied for their effects on the uniformity of additive distribution in granulations. Sixty-gram powder mixtures containing 0.5% by weight of the labeled phosphate salt in lactose were separately massed in a Mixmaster mixer at speed 2 for three minutes with three different granulating agents, pressed through a number 20 sieve, and oven dried at 50°. The granulating agents used were: water, a solvent for the labeled phosphate additive; dilute alcohol U. S. P., a partial solvent; and alcohol U. S. P., a nonsolvent for the additive. The results of this experiment are shown in Table II.

No significant difference in uniformity of distribution between powder and granulation occurs except in granulation 1 as can be seen from a comparison of $V\%$ values of Table II. Therefore, distribution of additives in granulations can be improved over that of the parent powder when: (*a*) the additive is sufficiently soluble in the granulating agent and (*b*) an adequate amount of granulating agent is added (note no change in distribution in

TABLE I.—THE EFFECT OF VARIOUS PARTICLE SIZE DISTRIBUTIONS OF ADDITIVE AND EXCIPIENT ON POWDER UNIFORMITY

Powder Number	$KH_2P^*O_4$ Fraction[a]	Lactose Fraction[a]	Min. Mixed	$V\%$[b]	Significance of Difference at 5% Level[c]
1	80/100	80/100	15	115.6	No—any combination of
2	100/150	100/150	15	135.0	1, 2, and 3
3	150/200	150/200	15	172.6	Yes—1 and 4, 2 and 4, and
4	Through 200	Through 200	15	17.3	3 and 4
5	80/100	Through 200	3	27.1	No—any combination of
6	80/100	Through 200	15	41.8	5, 6, and 7
7	80/100	Through 200	30	42.5	
8	100/150	Through 200	3	25.1	No—any combination of
9	100/150	Through 200	15	22.3	8, 9, and 10
10	100/150	Through 200	30	38.5	
11	150/200	Through 200	3	25.4	No—any combination of
12	150/200	Through 200	15	24.6	11, 12, and 13
13	150/200	Through 200	30	14.9	
14	Through 200	Through 200	3	51.2	No—15 and 16
15	Through 200	Through 200	15	17.3	Yes—14 and 15
16	Through 200	Through 200	30	19.8	14 and 16
17	Through 200	100/150	15	59.5	No—17 and 18
18	Through 200	150/200	15	36.2	Yes—18 and 19
19	Through 200	Through 200	15	17.3	17 and 19

[a] The sieve separated powder fractions were designated by ratio expressions in which the numerator indicates the last sieve number the powder passed through and the denominator indicates the sieve on which the powder was retained.

[b] $V\%$ is the per cent coefficient of variation (6). $V\% = (s/\bar{X})\ 100$, where s is the standard deviation of ten samples and $\bar{X}$ is the mean.

[c] To determine the significance of difference between the coefficients of variation of the different powder mixtures the test statistic, $V_1 - V_2/\sigma_{V_1 - V_2}$, was compared to the normal curve standard score at the 5% level (7). V_1 and V_2 are the two coefficients of variation being compared and $\sigma_{V_1 - V_2} = \sqrt{V_1^2/2n_1 + V_2^2/2n_2}$, where n is the number of samples (ten or more) on which the uniformity of activity of each tablet matrix was determined. This method assumes the ratio $V_1 - V_2/\sigma_{V_1 - V_2}$ to be normal and is valid when it is predicted before an experiment which comparisons are to be made. "Yes" indicates that there is a significant difference in the coefficients of variation being compared. "No" indicates that this difference is not statistically significant.

granulation 4). This would imply that when the two conditions above are met, the uniformity of distribution of additive material in a powder mixture may be of secondary importance in obtaining a uniform distribution of additive in a granulation.

Three-hundred and fifty gram fractions of a powder mixture of lactose and labeled phosphate salt were separately massed five minutes in a Mixmaster mixer at speed 2 with water, dilute alcohol U. S. P., and alcohol U. S. P. Each powder mass was divided into seven equal parts, dried by a number of methods, and the uniformity of distribution determined as shown in Table III.

With the exception of granulation 11, the granulations of Table III prepared with dilute alcohol and dried at a temperature exceeding 50° (granulations 10, 12, 13, and 14), were significantly less uniform than the corresponding water granulations (granulations 3, 5, 6, and 7). All of the alcohol granulations were significantly less uniform than the corresponding water granulations. The granulations prepared with the partial solvent granulating agent of dilute alcohol exhibited the greatest variation in uniformity depending on the method of drying employed, indicating that additive migration may have occurred at the higher drying temperatures. Continually stirring the granulations by hand as they dried under infrared lamps (granulations 7, 14, and 21) produced no significant increase or difference in the uniformity of additive dispersion over the corresponding granulations which were not stirred (granulations 6, 13, and 20).

Autoradiographs were prepared of a number of the granulations shown in Table III (Fig. 1), which varied in uniformity as expressed by the coefficients of variation. The granulations to be autoradiographed were distributed over Scotch brand cellophane tape and exposed to Eastman No-screen

TABLE II.—THE UNIFORMITY OF WATER, DILUTE ALCOHOL, AND ALCOHOL GRANULATIONS OF MONOBASIC POTASSIUM PHOSPHATE AND LACTOSE POWDER MIXTURES

	Powder or Granulation[a]	Ml. of Granulating Agent Used Per 100 Gm. of Powder	$V\%$[b]	Significance of Difference Between $V\%$ of Granulation and Parent Powder, 5% Level
I.	First Powder Mixture	..	13.5	..
1.	Water Granulation	8.0	6.8	Yes
2.	Dil. Alcohol Granulation	11.2	12.7	No
3.	Alcohol Granulation	25.0	18.6	No
II.	Second Powder Mixture	..	16.0	..
4.	Water Granulation	6.5	13.4	No
5.	Dil. Alcohol Granulation	10.0	14.5	No
6.	Alcohol Granulation	27.5	13.9	No

[a] The first powder mixture was mixed thirty minutes and the second was mixed ten minutes. Three granulations were made of each powder mixture using granulating agents of water, dilute alcohol U. S. P. and alcohol U. S. P., oven drying the granulations at 50°.

[b] The per cent coefficient of variation (Table I, footnote *b*) in each case was based on a sample size of twelve instead of ten.

TABLE III.—THE EFFECT OF THE DRYING METHOD ON THE UNIFORMITY OF DISTRIBUTION OF LABELED MONOBASIC POTASSIUM PHOSPHATE IN TABLET GRANULATIONS

Granulation Number	Granulating Agent	Method of Drying	$V\%$[d]	Significance of Difference at the 5% Level[f]
1	Water	Air[a]	13.8	Yes—2 and 7
2	Water	Oven 50°	15.9	No—all other combinations of 1 through 7
3	Water	Oven 75°	10.6	
4	Water	500 Watts I. R.[b]	9.9	
5	Water	375 Watts I. R.	13.7	
6	Water	250 Watts I. R.	10.7	
7	Water	250 Watts I. R.[c]	8.8	
8	Dil. Alcohol	Air	19.1	Yes—8 and 10
9	Dil. Alcohol	Oven 50°	27.7	Excluding 11; no—all other combinations of 8 through 14
10	Dil. Alcohol	Oven 75°	44.5	
11	Dil. Alcohol	500 Watts I. R.[c]	14.0	
12	Dil. Alcohol	375 Watts I. R.	35.8	
13	Dil. Alcohol	250 Watts I. R.	31.0	
14	Dil. Alcohol	250 Watts I. R.[c]	24.6	
15	Alcohol	Air	34.0	No—all combinations of 15 through 21
16	Alcohol	Oven 50°	36.9	
17	Alcohol	Oven 75°	35.1	
18	Alcohol	500 Watts I. R.	41.6	
19	Alcohol	375 Watts I. R.	25.8	
20	Alcohol	250 Watts I. R.	26.6	
21	Alcohol	250 Watts I. R.[c]	39.3	

[a] Air drying was at room temperature, 25°.

[b] The infrared lamps were used in pairs in a Fostoria evenray twin lamp holder at a ten-inch lamp to object distance. The maximum temperatures recorded ten inches from the infrared lamps were: 70° with the 250 watt lamps; 80° with the 375 watt lamps; and 95° with the 500 watt lamps.

[c] These granulations were constantly stirred by hand as they dried.

[d] The per cent coefficient of variation (Table I, footnote *b*) for granulations 1–7 was based on a sample size of fourteen and for granulations 8–21 on a sample size of ten.

[e] The low per cent coefficient of variation of granulation 11 was unexplained. Autoradiographs roughly substantiated this finding.

[f] See Table I, footnote *c* for the test for significance of difference.

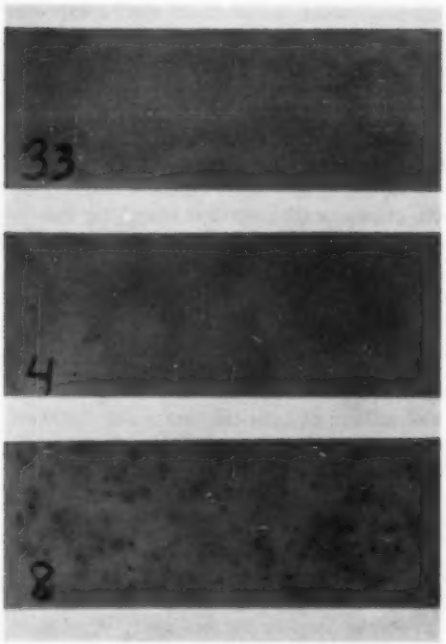

Fig. 1.—Granulation Autoradiographs. Granulation number 33 had a coefficient of variation of 4.0%, granulation number 4 (Table III) had a coefficient of variation of 9.9%, and granulation number 8 (Table III) had a coefficient of variation of 19.1%.

X-ray film. Gross visual examination of the granulation autoradiographs indicated complete agreement with the per cent coefficient of variation obtained by the statistical method.

Separate powder mixtures of lactose and labeled phosphate salt were moistened with water and the moistened powders mixed or massed for periods of two and one-half and twenty-five minutes. The per cent coefficient of variation of the resulting granulations was 18.1 and 20.7% respectively. The difference in the additive uniformity of distribution of the two granulations, depending on the duration of the massing time employed, was not statistically significant at the 5% level.

The water-soluble labeled phosphate additive was dissolved in water, and this solution used as the granulating agent for the lactose excipient. The granulations were air dried at room temperature, oven dried at 50°, and infrared dried at 500 watts, ten inches. The per cent coefficients of variation of these granulations was 7.0, 2.9, and 3.6% respectively. At the 5% level the granulations prepared by dissolving the additive in the granulating agent were significantly more uniform than the corresponding granulations of Table III (granulations 1, 2, and 4) which were prepared by adding the same concentration of granulating agent to the powder mixture of additive and excipient.

The effect on additive uniformity of distribution of the various methods of drying an alcohol and water-soluble additive, phosphoric acid, using the different granulating agents, was studied. The results are shown in Table IV.

When an alcohol solution of the additive was used as the granulating agent (granulations 22–28) infrared drying with the 500-watt lamps (granulation 25) resulted in a granulation significantly less uniform than the other alcohol granulations. Migration of the additive evidently occurred at the temperature of the 500-watt infrared drying method (95°) but was insignificant at the lower temperatures of the other drying methods. Granulation 25 was recounted and the original results were verified. The granulations prepared using a granulating agent of water or dilute alcohol showed no significant migration. Mixing of the dried granulations which had apparently not undergone appreciable migration effects (granulations 22–24 and 26–36) did not significantly increase the uniformity of these granulations. Mixing of dry, sized granulation 25 which had apparently suffered migration effects did significantly increase the uniformity of additive dispersion over that of the same dry, sized granulation before mixing. The per cent coefficient of variation

TABLE IV.—THE EFFECT OF THE DRYING METHOD ON THE UNIFORMITY OF DISTRIBUTION OF AN ALCOHOL AND WATER-SOLUBLE ADDITIVE IN TABLET GRANULATIONS

Granulation Number	Granulating Agent	Method of Drying	V%[b]	Significance of Difference at 5% Level[c]
22	Alcohol	Air	6.6	
23	Alcohol	Oven 37°	6.5	No, 24 or 28 *vs.* 25
24	Alcohol	Oven 50°	7.3	
25	Alcohol	500 Watts I. R.	13.1	Yes, 22, 23, 26 or 27 *vs.* 25
26	Alcohol	375 Watts I. R.	6.0	
27	Alcohol	250 Watts I. R.	6.3	No, other combinations of 22 through 28
28	Alcohol	250 Watts I. R.[a]	7.7	
29	Dil. Alcohol	Oven 37°C.	2.7	No significant difference between granulations 29 through 32
30	Dil. Alcohol	Oven 75°C.	2.6	
31	Dil. Alcohol	500 Watts I.R.	1.6	
32	Dil. Alcohol	250 Watts I.R.	1.8	
33	Water	Oven 37°	4.0	No significant difference between granulations 33 through 36
34	Water	Oven 75°	2.8	
35	Water	500 Watts I.R.	2.8	
36	Water	250 Watts I.R.	3.4	

[a] The granulation was constantly stirred by hand as it dried.
[b] The per cent coefficient of variation (Table I, footnote *b*).
[c] See Table I, footnote *c* for the test for significance of difference.

of granulation 25, after the granulation had been tumble mixed for five minutes was 4.8%. Immediately after drying and before mixing the per cent coefficient of variation of the same granulation as shown in Table IV was 13.1.

Some of the granulations in Tables III and IV were separated into fines (pass 60 mesh) and granules (retained on 60 mesh). These were counted (ten 10.0-mg. samples of each fraction) to see if the labeled phosphate additive had a different concentration in the fines and granules. A "t" value was calculated from the corrected average activity of the fine and granule samples and a 1% significance level was used to see if the differences found were significant. On this basis a real difference in concentration of additive was found between fines and granules for the dilute alcohol and alcohol granulations of Tables III and IV studied, the granule fraction in every case being the more active (Nos. 12–18 and 25). No significant difference was found between fine and granule activity of the water granulations studied. Explanation of these findings will require additional work.

Labeled phosphate salt granulations prepared with granulating agents of water, dilute alcohol, and alcohol, and varying considerably in uniformity expressed as the per cent coefficient of variation (6.8 to 22.6%), were lubricated with 1.5% magnesium stearate and compressed into $^3/_8$-inch tablets on a Stoke's Eureka hand tablet machine. In every case there was no significant difference in additive uniformity of distribution between ten 10.0-mg. samples from each tablet and the granulation from which the tablet was prepared. Some of the per cent coefficients of variation compared were: 8.0 *vs.* 6.8; 8.2 *vs.* 12.7; 18.9 *vs.* 18.6; 15.3 *vs.* 22.6; and 17.0 *vs.* 13.9. Tablet autoradiographs of $1^1/_8$ inch diameter tablets prepared on a Carver laboratory press provided substantiation on the basis of gross visual examination of the statistical method of determining tablet uniformity of additive distribution.

SUMMARY

A method employing radioactive tracer techniques for studying medicament distribution in powder mixtures, granulations, and tablets has been developed and found to be a useful tool in such studies.

1. Powders consisting of phosphorus-32 labeled monobasic potassium phosphate as additive and lactose powder U. S. P. as excipient were prepared. The uniformity of distribution of the labeled phosphate was affected by the particle size distribution of additive and excipient and by the mixing time. Best uniformity was obtained with 150/200 or 200 mesh phosphate and 200 mesh lactose tumble mixed for fifteen minutes.

2. The void volume of the lactose excipient had no effect on the uniformity of distribution of the phosphate.

3. The uniformity of drug distribution in a tablet is the same as in the starting powder unless a drug-soluble granulating agent is used. The best uniformity achieved in this study was ±13.5% in a powder. Thus individual tablets made from this powder will vary as much as 27% in drug content. Using a drug-soluble granulating agent, the granulations varied as little as ±1.6% or no more than 3.2% in the drug content of the resulting tablets. The formulation and the mixing techniques employed in this study were chosen for the purpose of identifying some of the causes of variation of tablet dosage. Consequently the typical size of additive dosage variation encountered in this problem should not be considered as representative of the variation normally encountered in tablet manufacture.

4. When using a granulating agent in which the additive material was soluble, significantly more uniform granulations were usually obtained when the additive was dissolved in the granulating agent than when the granulating agent was added to the powder mixture of additive and excipient.

5. Increasing the massing time beyond the few minutes necessary to uniformly wet the powder mixture with the granulating agent did not significantly increase the uniformity of additive dispersion.

6. The water-soluble, alcohol-insoluble labeled phosphate salt additive when granulated with water or alcohol produced granulations which did not vary significantly in uniformity depending on the drying methods employed (air drying at room temperature, oven drying below 75° or infrared drying below 95°). When this additive in a lactose powder mixture was granulated with dilute alcohol the air dried granulation was significantly more uniform than the granulation oven dried at 75°.

7. The water- and alcohol-soluble labeled phosphoric acid additive, when granulated with water or dilute alcohol produced granulations which did not vary significantly in uniformity due to the drying methods employed. The granulations prepared with an alcohol solution of phosphoric acid additive as the granulating agent did vary significantly in uniformity of additive dispersion depending on the method of drying employed. At the 5% significance level infrared drying at 95° produced a granulation significantly less uniform than infrared drying at 80° or below, oven drying at 37° or below or air drying at room temperature. Stirring the granulations as they dried under infrared lamps, regardless of the granulating agent or additive employed, did not increase the uniformity of additive dispersion over that of granulations not stirred.

8. The concentration of labeled phosphate

additive in alcohol and dilute alcohol granulations was different between material retained on a 60-mesh screen (granules) and material passing a 60-mesh screen (fines). This was not the case when water was the granulating agent.

9. The uniformity of additive dispersion within compressed tablets was found to be statistically identical to the degree of uniformity of additive distribution of the granulations from which the tablets were made.

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Potentiometric and Amperometric Titrations of Ascorbic Acid*

By S. M. DESHPANDE and R. NATARAJAN

Potentiometry and amperometry using a dropping mercury electrode have been tried and found suitable for the estimation of ascorbic acid by titration with potassium permanganate. It has been confirmed that there is a necessity for the addition of potassium iodide to get the end point at the dehydroascorbic acid stage. Quantities less than 1 mg. of ascorbic acid could be estimated potentiometrically within ± 1.5 per cent deviation. The possibility by amperometry, of other permanganometric titrations using potassium iodide as indicator, has been suggested.

THE 2,6-DICHLOROPHENOL-INDOPHENOL METHOD (1) for the estimation of ascorbic acid has certain inherent defects, such as the instability of the dye solution necessitating standardization often, and the transient nature of the end point which introduces considerable difficulties. In an attempt to find a suitable alternate method, the permanganometric method of Murti and Viswanadham (2) was considered. They have reported that the end point in lower concentrations of ascorbic acid was achieved at equivalent proportions only by use of starch-potassium iodide as an indicator; furthermore, a deviation of +2.64% in titrations using 0.001 *N* permanganate for amounts less than 1 mg. of ascorbic acid, has been reported. Here, the permanganate is expected to first liberate iodine from potassium iodide, which in turn reacts with ascorbic acid to give dehydroascorbic acid. It was therefore thought of confirming the necessity for the addition of potassium iodide, by other methods. Attempt has also been made to see whether the error could be minimized by any other suitable method.

EXPERIMENTAL

Chemicals of AR grade and ascorbic acid (E. Merck) which was standardized by iodimetric method (3) were used in these experiments. Further, the use of conductivity water as recommended by Murti and Viswanadham for the permanganate method was considered not practicable for routine analysis and therefore ordinary distilled water was employed. Ascorbic acid solutions were prepared as and when required, by taking requisite amount in 100 ml. of water containing 4 ml. of 2 *N* sulfuric acid as stabilizing agent; such solutions were found to be stable for about two hours at room temperature (25 ± 2°).

Potentiometry.—Pye's "S" model which gives an accuracy of 1 mv. and a constant stirrer assembly for mixing the solution were used. One ml. of ascorbic acid solution was taken in a titration vessel of 100 ml. capacity, with 2 ml. of 2 *N* sulfuric acid and 1 ml. of 0.5% potassium iodide solution along with 50 ml. of water. This solution was titrated with 0.001 *N* permanganate (except where indicated, see Table I) using a platinum wire electrode as the indicator and saturated calomel electrode as the reference electrode. The E. M. F. of the solution after one minute of each addition of permanganate was noted by the null deflection method; the titration was continued up to about 2 ml. excess addition of permanganate after the end point, as usual. The equivalence point for each titration was determined by plotting $\Delta E/\Delta V$ against volume of permanganate added; a typical curve obtained is given in Fig. 1.

Amperometry.—The polarographic equipment used for this purpose was made by Messrs. Adept Laboratories, Poona, India; this was used in conjunction with a spot type suspension coil galvanometer and a dropping mercury electrode.

In the polarographic cell, a solution containing 1 ml. of a solution of ascorbic acid, 2 ml. of 2 *N* sulfuric acid, 1 ml. of 0.5% potassium iodide, and 40 ml. of water was taken. The dropping mercury

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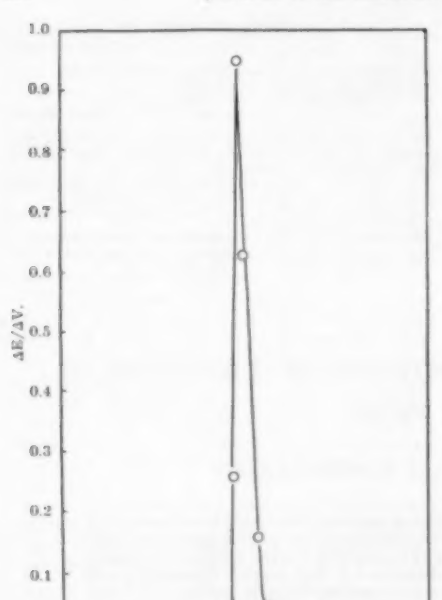

Fig. 1.—Potentiometric determination of ascorbic acid (0.5 mg.).

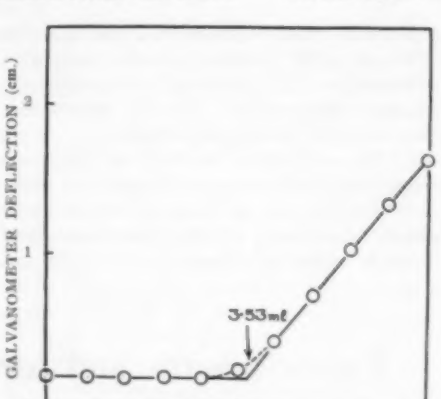

Fig. 2.—Amperometric determination of ascorbic acid (3.0 mg.).

electrode was dipped into the solution so as to be 1 cm. below the surface of the liquid; and the mercury level-head was adjusted to give a drop time of 3.33 seconds. A constant potential of −0.1 volt *vs.* S. C. E. was applied and the titration with permanganate (0.01 *N*) was carried out noting the deflection of the galvanometer after each addition. The galvanometer deflection was plotted against volume of permanganate added, to get the end point; a typical curve obtained is given in Fig. 2.

RESULTS AND DISCUSSION

Potentiometry.—When ascorbic acid was titrated against potassium permanganate, a sharp end point could not be obtained; the galvanometer deflections were not steady, but showed a tendency to fall back to the original level in about a minute. This is because the reaction does not stop at the dehydroascorbic acid stage as reported by Herbert, *et al* (4). However, the addition of potassium iodide above 1 ml. of 0.5% brought about a sharp end point at the dehydroascorbic acid stage indicating that the permanganate liberates iodine from potassium iodide which, in turn, oxidizes the whole ascorbic acid, and finally the free iodine produces the change of potential. In any case, the reaction has been found suitable for potentiometric determination of small quantities of ascorbic acid by titration with even 0.001 *N* permanganate in presence of potassium iodide.

The presence of potassium iodide in the range of 1 ml. of 0.5 to 8% solution gave consistent values for the end point. Similarly, sulfuric acid concentration could be altered between 1 and 5 ml. of 2 *N* strength without affecting the results. Therefore, for convenience, 1 ml. of 0.5% potassium iodide and 2 ml. of 2 *N* sulfuric acid as recommended by Murti and Viswanadham were employed and the results thus obtained for various concentrations of ascorbic acid are given in Table I. In addition, a comparison has been made of the results obtained by potentiometry with the values obtained by direct titration using 1 ml. of 0.5% starch as indicator.

Table I.—Assay of Ascorbic Acid

Ascorbic Acid Taken, mg.	By Potentiometry: Ascorbic Acid Found, mg.	By Potentiometry: Percentage Deviation	By Direct Titration Using Starch as Indicator: Ascorbic Acid Found, mg.	By Direct Titration Using Starch as Indicator: Percentage Deviation
0.30	0.302	+0.67	0.289	−3.60
0.50	0.495	−1.00	0.503	+0.60
0.80	0.789	−1.37	0.813	+1.63
1.00	0.990	−1.00	0.996	−0.40
3.00[a]	3.018	+0.60	3.018	+0.60
4.00[a]	3.983	−0.43	4.065	+1.60
6.00[a]	6.037	+0.61	6.076	+1.27

[a] 0.01 *N* permanganate was used; for the others 0.001 *N* permanganate.

Amperometry.—Ascorbic acid in sulfuric acid medium does not give any polarographic wave. Potassium permanganate is reported to give no polarographic wave suitable for analytical purpose (5). Therefore, the amperometric titration of ascorbic acid with permanganate directly is not possible. The permanganometric method using potassium iodide can, however, be made the basis of an amperometric method.

The deviation obtained in potentiometry in the

region of lower concentrations of ascorbic acid is of the order of ±1.5%.

From preliminary experiments it was found that consistent values were obtainable at an applied potential of −0.1 volt *vs.* S. C. E. in this method. Accordingly, various amounts of ascorbic acid were titrated, and the results are given in Table II, along with the values obtained by direct titration.

TABLE II.—AMPEROMETRIC DETERMINATION OF ASCORBIC ACID

Ascorbic Acid Taken, mg.	—By Amperometry— Ascorbic Acid Found, mg.	Percentage Deviation	By Direct Titration Using Starch as Indicator Ascorbic Acid Found, mg.	Percentage Deviation
1.0	0.995	−0.50	1.012	+1.2
2.0	2.007	+0.35	2.024	+1.2
3.0	3.002	+0.06	3.045	+1.5
4.0	3.973	−0.67	4.024	+0.6

Strength of pot. permanganate, 0.01 *N*. Applied potential, −0.1 volt *vs.* S. C. E. Galvanometer shunt, 1/2.

Results obtained by this method, however, showed that the amperometric method is not sensitive enough with 0.001 *N* permanganate. It is also evident that whenever permanganate is to be used in amperometric titrations, potassium iodide may serve as an indicator.

SUMMARY

The permanganometric method of titrating ascorbic acid in presence of potassium iodide and sulfuric acid has been tried by potentiometry and amperometry. Here potassium iodide acts as an indicator. The potentiometric method gives an error of ±1.5% for ascorbic acid less than 1 mg., whereas the amperometric method is not sensitive enough below 1 mg. of ascorbic acid.

It should be possible to extend this method for other permanganate titrations which are not normally possible by amperometry.

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Evaluation of Oral Antiseptic Products on Buccal Epithelial Tissue*

By LEONARD J. VINSON and ALLAN G. BENNETT

Two *in vivo* methods for evaluating antiseptic properties of oral products such as mouthwashes and dentifrices are described. Buccal tissue scrapings removed by curettage serve as the test substrate in estimating oral bacterial count (Buccal Tissue Count Test) and in measuring the degree of affinity of the antiseptic to mouth tissue after brushing with a dentifrice or swishing with a mouthwash (Buccal Epithelial Substantivity Test). The test cultures employed were *M. pyogenes* var. *aureus* and *L. casei*. The buccal tissue shows inhibitory effect against oral bacteria lasting up to four hours after one treatment of a hexachlorophene-containing mouthwash or dentifrice.

DESPITE the large number of tests reported in the literature a reliable *in vivo* method is still needed for evaluating antiseptic oral products with particular reference to dentifrices and mouthwashes. Many factors appear to influence the type and numbers of the oral flora in the mouth. Saliva counts, for example, fluctuate widely during the course of a day being influenced by diet, by changes in salivation rate, and by environmental factors. Thus, counts made on stimulated or unstimulated saliva are poorly reproducible and do not serve as a good basis for evaluating oral antiseptics (1). The rinse technique as described by Ostrolenk and co-workers (2) and Watson and Reddish (3) is an improvement, but the shortcomings of the saliva count method remain.

The swab technique as described by Bloomfield (4) and many modifications thereof employ oral surfaces such as teeth, tongue, buccal epithelium, and palate. Again, the wide variability in counts encountered makes it a poor choice for evaluating oral antiseptic agents.

As indicated by Ostrolenk and co-workers (2) the evaluation of the effectiveness of oral antiseptics must primarily rely upon securing representative samples of the oral microbial population before and after use of the product. It is the purpose of this report to describe a new method for evaluating oral antiseptic agents using buccal

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epithelial scrapings from the mouth. These are examined for the presence of adsorbed antiseptic agent, and bacterial counts are obtained.

METHODS

Buccal Tissue Count Test.—Buccal epithelial scrapings are readily obtained from a subject's mouth by means of a curette.[1] This is done simply and easily by the subject himself, who gently scrapes the inside of either cheek using several strokes until the cuplike receiver of the curette is filled with a mixture of mucus-epithelial detritus. With a little experience the amount removed is readily standardized as reflected in the reproducible counts obtained. The tissue is transferred from the curette by agitation into 10 ml. sterile 0.1% peptone water contained in a screw-capped test tube. The contents are shaken thoroughly, diluted further when necessary, and 1-ml. aliquots in duplicate plated directly into Blood Agar Base (Difco). Counts are made after forty-eight hours incubation at 37°.

Buccal Epithelial Substantivity Test.—Buccal epithelial scrapings are removed from the mouth as described previously. The tissue is scooped out of the curette with the tapered end of a metal spatula and transferred to the center of the filter paper disk.[2] The disks with the flattened tissue are allowed to dry for one hour at 55° and then placed tissue side down on seeded agar plates. In the case of the test organism, *M. pyogenes* var. *aureus*,[3] Nutrient Agar is employed and for *Lactobacillus casei*[4] the medium is Tryptone Glucose Extract (TGE) Agar with 2% added yeast extract. A number of disks can be placed peripherally (3/4 inch from the edge) on the agar surface. Additional disks can be situated in the center area. Thus, buccal tissue specimens from one or more subjects can be conveniently placed on the same seeded agar plate.

The plates are incubated overnight at 37°. The disks are removed and the zones of tissue contact with agar are examined microscopically for evidence of bacterial colonies, focusing at the surface. This is accomplished by bringing into sharp resolution the imprint of the disk and epithelial tissue on agar. By employing a reticule (Howard micrometer disk) the colonies in four squares picked at random in the central contact region are counted and a similar count is made in an adjacent agar area not in contact with the buccal scrapings (control counts). The per cent reduction in count of area in contact with the buccal tissue is determined on the basis of the control count.

Buccal Tissue Count Test Results.—The antiseptic action of dentifrices and mouthwashes on oral flora is evaluated by running buccal tissue counts before and after use of the products.

The reproducibility of counts was determined on replicate samples of buccal tissue from each of eight subjects. Results are given in Table I.

The replicate series yield counts showing good reproducibility. Fluctuations do occur between the average value of a series and that obtained at a different time during the day on the same subject. The better reproducibility in average counts made on buccal scrapings is due to the use of replicate samples. It is not possible to obtain replicates of saliva at one sampling period, since successive saliva samples would be representative of a changing oral condition as fresh saliva is secreted to replace that taken in sampling. With the buccal scrapings technique, four essentially simultaneous samples can be taken without disturbing the salivation rate. Representative buccal count data obtained on three subjects after single brushings with plain and antiseptic dentifrices, and with water are shown in Table II. A decided drop in buccal count occurred after brushing with the antiseptic dentifrice. The individual final counts in every instance fell considerably below even the lowest initial value. This was not observed when the subjects brushed with an ordinary dentifrice, the reductions in count being only moderate. Subjects on the water control showed no reductions in count.

The apparent increase in buccal counts after a water wash is not real and is attributable to the fact that a more diligent scraping over a wider area of the buccal tissue is required to obtain a full sample in the curette. The fact that this increase in count does not occur after use of a dentifrice or mouthwash is explained by the partial destruction of buccal bacteria on immediate contact with the preparation.

Dentifrices and mouthwashes were evaluated for antiseptic activity employing the buccal tissue count test. Included in this series were a toothpaste and a mouthwash containing hexachlorophene, at 0.05% and 0.02% levels, respectively. Twenty-three to thirty subjects were employed for each product and a water control. The procedure was standardized as follows: The subject brushed with the dentifrice or swished with the mouthwash once in the morning for a period of one minute followed by two five-second water rinses. Before and immediately after the oral treatment buccal scrapings were taken for bacterial counts in the prescribed manner.

TABLE I.—REPRODUCIBILITY OF BACTERIAL COUNTS OF REPLICATE BUCCAL TISSUE SCRAPINGS[a]

Buccal tissue replicate	Subjects							
	1	2	3	4	5	6	7	8
a	52,160	48,420	132,480	123,840	2,740	53,020	16,000	8,640
b	46,450	56,000	115,200	103,680	5,120	89,280	15,630	16,000
c	39,680	66,780	69,120	123,840	2,880	70,080	16,960	7,320
d	60,380	57,820	135,360	98,000	5,120	74,880	18,240	12,800
Mean	49,668	57,255	113,040	112,340	3,965	71,815	16,708	11,190

[a] The scrapings collected in a curette are obtained after several contact strokes on the inside of the mouth (buccal tissue). The area samples represent a small fraction of the mouth tissue.

[1] Mayhoefer chalazion curette, No. 3.
[2] Whatman #2, 12.7 mm. diameter.
[3] Twenty-four hour culture in Nutrient Broth (Difco)—1% Inoculum.
[4] Twenty-four hour culture in Microinoculum Broth (Difco)—1% Inoculum.

Two hours later repeat buccal tissue scrapings were removed for analysis. Average per cent reductions in bacterial counts are given in Table III.

count rose after two hours to a value actually higher than the initial. The water control was without effect in the buccal tissue count test.

TABLE II.—EFFECT OF SINGLE BRUSHINGS WITH AN ANTISEPTIC DENTIFRICE, PLAIN DENTIFRICE, AND WATER ON BUCCAL TISSUE COUNTS (4 REPLICATES)

Subject[a]		Water		Plain Dentifrice		Antiseptic Dentifrice	
		Before	After	Before	After	Before	After
1		2,740	6,400	9,280	3,140	10,160	460
		5,120	8,960	11,520	1,500	11,280	1,620
		2,880	17,280	11,200	1,650	8,480	210
		5,120	6,400	11,200	9,920	9,280	120
	Mean	3,965	9,760	10,725	4,053	9,800	603
2		53,020	66,240	8,640	1,030	34,130	280
		89,280	86,400	7,360	2,150	33,760	340
		70,080	67,200	8,320	7,360	30,390	360
		74,880	77,760	8,320	7,040	9,920	430
	Mean	71,815	74,400	8,160	4,395	29,278	353
3		8,640	30,080	13,760	2,940	33,240	1,980
		16,000	23,360	20,480	5,350	39,620	920
		7,320	26,880	15,360	4,480	39,960	1,060
		12,800	27,520	23,680	5,440	43,050	1,020
	Mean	11,190	26,960	18,320	4,553	38,968	1,245

[a] Brushings with dentifrices and water made on different days.

TABLE III.—PER CENT REDUCTIONS IN BACTERIAL COUNTS OF BUCCAL TISSUE SCRAPINGS FOLLOWING A SINGLE ORAL TREATMENT[a] WITH COMMERCIAL DENTIFRICES AND MOUTHWASHES

	No. of Subjects	Antiseptic Component	Antibacterial Effectiveness			
			Immediately After		2 Hours Later	
			Av. % Reduction	Reversals,[b] %	Av. % Reduction	Reversals, %
Dentifrice:						
A	27	0.05% Hexachlorophene	94	0	45	11
B	26		49	12	3	54
C	28		35	25	−20	60
D	28		52	10	−36	78
E	30		37	27	−14	53
Mouthwash:						
F	28	Thymol; boric and benzoic acid; alcohol	61	14	−24	64
G	23	0.02% Hexachlorophene; alcohol	95	0	30	17
Water	26	.....	−22	77	−26	69

[a] The subject brushed with a dentifrice for one minute followed by two five-second rinses with water. With the undiluted mouthwash or water control, the subject swished 10–20 ml. sample for 1 minute, followed by two five-second rinses with water.
[b] A reversal refers to a buccal tissue count higher after treatment than before.

From the results, it is evident that the dentifrices and mouthwashes act to give an immediate reduction in buccal tissue count. Dentifrice A with 0.05% hexachlorophene gave an average reduction in bacterial count of 94%. This was significantly better than the other dentifrices. The reduction in buccal tissue count effected by Dentifrice A was still evident two hours after the single treatment. The other products did not show any residual effect. The outstanding action of Dentifrice A is also apparent in comparing the reversals (increase in count) for the different preparations. With the dentifrice containing hexachlorophene, there were no reversals immediately after treatment and a significantly lower number of reversals two hours later. Mouthwash G with 0.02% hexachlorophene gave an immediate reduction in buccal tissue count of 95% and the count remained depressed for at least two hours. Mouthwash F with thymol and other ingredients showed a 61% immediate reduction but the average

Buccal Epithelial Substantivity Test Results.—The ability of dentifrices and mouthwashes to show a residual antiseptic effect on oral flora after brushing or swishing was investigated employing the Buccal Epithelial Substantivity Test described previously. Buccal tissue scrapings were removed from subjects' mouths in the designated manner prior to a single treatment[5] with an oral product. Immediately after use of the product, and one, two, and four hours later, additional samples were taken. Duplicate samples of buccal tissue scrapings for each period were evaluated for their inhibitory effect, one against *M. pyogenes* var. *aureus* and the other against *L. casei*. Per cent reductions in bacterial counts are recorded in Table IV.

The results give convincing evidence of the affinity of hexachlorophene to buccal tissue of subjects' mouths treated with Dentifrice A containing 0.05%

[5] One minute brushing or swishing followed by two five-second rinsings.

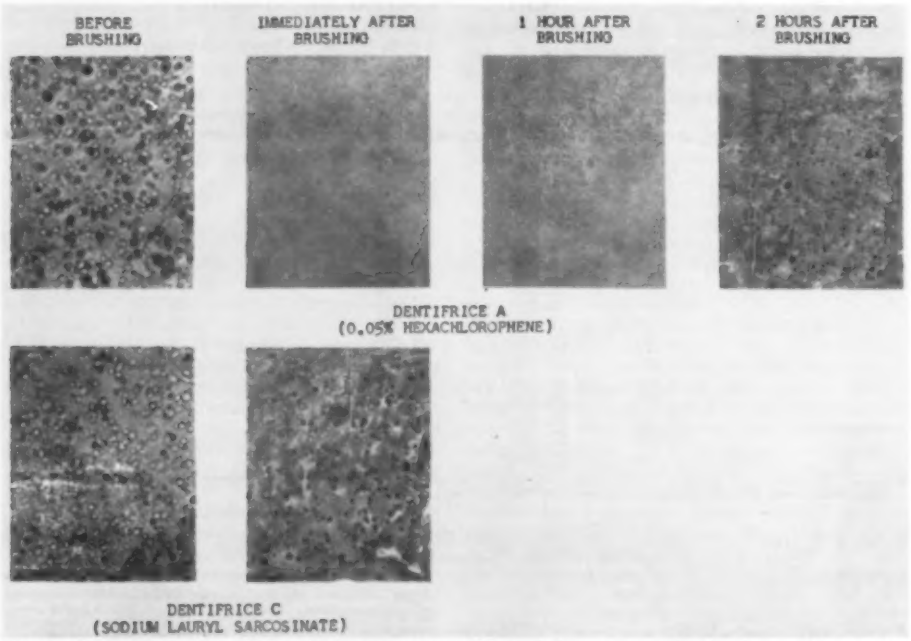

Fig. 1.—Photomicrographs (175 ×) of contact areas of buccal tissue to agar showing the sustained inhibitory effect of dentifrice A against *Lactobacillus casei*. Dentrifrice C is without effect.

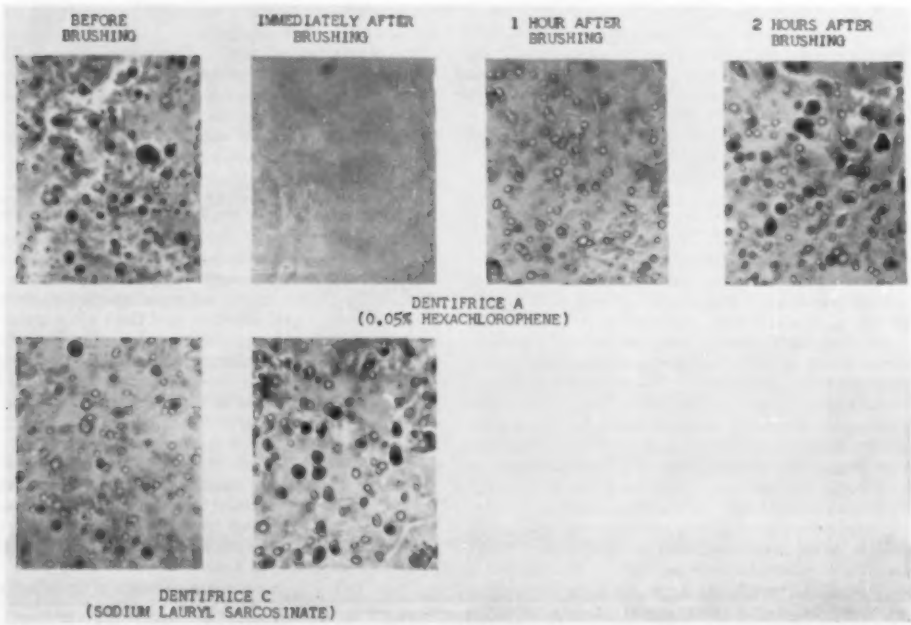

Fig. 2.—Photomicrographs (175 ×) of contact areas of buccal tissue to agar showing the sustained inhibitory effect of dentifrice A against *M. pyogenes* var. *aureus*. Dentifrice C is without effect.

TABLE IV.—BUCCAL EPITHELIAL SUBSTANTIVITY DATA FOR DENTRIFICES AND MOUTHWASHES

	Antiseptic Ingredient	Reduction in Bacterial Counts of Contact Area, %									
		Before Treatment		Immediately After		1 Hour Later		2 Hours Later		4 Hours Later	
		M[a]	L[b]	M	L	M	L	M	L	M	L
Dentifrice:											
A	0.05% G-11	<10	<10	100	100	73	74	55	46	<10	32
B		<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
C		<10	<10	27	10	<10	<10	<10	<10	<10	<10
D		<10	<10	<10	<10	15	<10	<10	<10	<10	<10
Mouthwash:											
F	Thymol; boric and benzoic acid; alcohol	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
G	0.02% G-11; alcohol	<10	<10	100	100	75	100	46	77	21	23
Saline	.....	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

[a] M denotes *Micrococcus pyogenes* var. *aureus*. [b] L denotes *Lactobacillus casei*.

hexachlorophene or Mouthwash G with 0.02% hexachlorophene. Immediately after brushing with the paste or swishing with mouthwash the buccal tissue develops strong antibacterial activity, as indicated by the complete inhibition of bacterial growth. The inhibitory nature of the buccal tissue persists at least up to four hours after the treatment. Ordinary dentifrices and Mouthwash F show little or no effect in developing an antibacterial mantle on buccal tissue. Saline shows no effect in this test.

Photomicrographs of seeded agar surfaces exposed to buccal tissue scrapings are shown in Figs. 1 and 2. The areas shown contrast the action of the hexachlorophene dentifrice which imparts strong antibacterial activity, with that of an ordinary dentifrice showing no activity.

DISCUSSION

Two reliable methods employing buccal tissue for evaluating the antiseptic properties of oral products like dentifrices and mouthwashes are described. Wide fluctuations in counts are not the rule as observed in the older methods employing saliva as the substrate.

The reduction in buccal tissue counts is considered a sound measure of the effectiveness of oral antiseptic products since the tissue is constantly exposed to bacteria-rich saliva. Buccal tissue counts, in a sense, reflect the oral cavity environment with respect to type and number of bacteria. It is, therefore, noteworthy that the buccal count remains relatively low in the presence of a tissue-substantive antiseptic like hexachlorophene in a mouthwash or dentifrice.

Hexachlorophene is known to be a potent bacteriostatic agent at very low concentrations. Ennever and co-workers (5) reported that growth of oral bacteria (pooled human saliva) and resultant acid production were completely inhibited in a dextrose-peptone medium containing as low as 0.125 p. p. m. hexachlorophene.

Florestano and co-workers reported (6) that organisms such as streptococci, staphylococci, pneumococci, lactobacilli, fusiform bacillus, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Borrelia vincenti*, and other spirochetes are constantly found in the mouth both in health and disease. They point out that the mouth tissue cannot be disinfected and the only practical approach to control potentially harmful oral microorganisms is to keep down their numbers.

The action of oral products in controlling bacterial growth in the mouth is readily assessed by the buccal tissue count test. The ability of an antiseptic in an oral product to cling to the mouth tissues and to continue to act for a sustained period is readily demonstrated in the buccal epithelial substantivity test. In the cases of a hexachlorophene-containing dentifrice or mouthwash, the buccal epithelial substantivity test data show a persistence of the antiseptic on buccal tissue at least up to four hours after a single oral treatment.

The buccal epithelial substantivity test can be modified by employing other test organisms to determine the activity of the oral products against a wide spectrum of bacteria. For example, in addition to testing *M. pyogenes* var. *aureus* and *L. casei*, other organisms such as those noted by Florestano (6) and even a heterogeneous oral flora culture from saliva can be used. Antibacterial activity for mouth tissue in the buccal epithelial substantivity test is a strong indication that bacteria on oral tissues *in vivo* are unable to multiply.

Substrates other than buccal tissue scrapings have also been used in assessing substantivity of antiseptics to oral surfaces. For example, tooth plaques can be removed from the mouth and shown to have an inhibitory effect against bacteria following oral treatment with the hexachlorophene-containing mouthwash or dentifrice.

The buccal tissue count test employing differential media is applicable in evaluating the effectiveness of antiseptic oral products with respect to the different bacterial species that comprise oral flora. For example, by plating out buccal scrapings samples into blood agar, the hemolytic streptococci and micrococci can be detected and counted. Hexachlorophene-containing dentifrice and mouthwash were found to be effective in reducing the buccal tissue counts of these hemolytic cocci. The reduction persisted for several hours.

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An Evaluation of the Pharmacologic Activity of a New Series of Chalcone Derivatives*

By G. VICTOR ROSSI and ELIAS W. PACKMAN

A series of fourteen new chalcone derivatives, synthesized as potential antagonists of norepinephrine biogenesis, were evaluated with regard to their hypotensive potency in the normotensive dog. The compound 2-(2-dimethylaminoethoxy) chalcone citrate, selected as the most active member of this group, was subjected to further study including determination of depressor effect in the hypertensive rat, acute toxicity, influence on epinephrine toxicity, and modification of barbiturate induced "sleeping time." This series of chalcones was also screened for antihistaminic and antispasmodic activity on isolated guinea pig ileum, and the three most effective compounds in this respect were examined for their ability to inhibit the histamine depressor response in the cat. The chalcones investigated in this study compared unfavorably with available therapeutic agents in regard to specificity, potency, and duration of action. Recommendation is made for synthesis of additional chalcones with consideration given to the activities reported.

CLARK (1) REPORTED in 1953 that certain hydroxychalcones inhibit 3,4-dihydroxyphenylalanine (dopa) decarboxylase. Presuming that active chalcones could be synthesized, this finding suggested the possibility of blocking the biogenesis of norepinephrine, the adrenergic neurohumoral transmitter. Such compounds might reasonably be expected to possess hypotensive activity.

Hydroxychalcones have been found to interfere with the activity of various enzyme systems. Beiler (2) has shown that these compounds inhibit the activity of choline acetylase. Certain substituted chalcones, notably 3,3′,4,4′-tetrahydroxychalcones, were found by Beiler and Martin to function as effective inhibitors of rat liver xanthine oxidase *in vitro* (3) and *in vivo* (4). Hydroxychalcones were reported by Bartlett (5) to inhibit succinoxidase activity, probably due to the formation of quinones which react with essential sulfhydryl groups of the enzyme.

On the basis of Clark's observations (1), Packman and Rubin (6) prepared a series of chalcone derivatives as potential antagonists of norepinephrine biosynthesis. This report is primarily concerned with an evaluation of the blood pressure reducing activity of these compounds. However, certain of the structural features of these chalcones also warranted investigation of their antihistaminic activity. Notably, the groups joined by ether linkage to the chalcone nucleus are disubstituted alkylamino moieties, forming a structural class to which many potent antihistamines belong. The terminal nitrogen is tertiary, with some of the compounds possessing the N-dimethyl group generally found to be optimal for antihistaminic activity.

* Received from the LaWall Memorial Laboratory of Pharmacology and Biochemistry, Philadelphia College of Pharmacy and Science, Philadelphia 4, Pa.

This study was supported in part by a research grant from the National Drug Company, Philadelphia 44, Pa.

EXPERIMENTAL

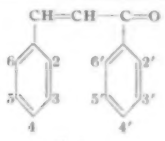
Of the original series of compounds synthesized by Packman and Rubin (6), those listed in Table I were available for pharmacologic evaluation.[1] Henceforth in this report the compounds will be referred to by the code letter assigned for the investigation.

Hypotensive Activity in Normotensive Dogs.—Fourteen chalcone derivatives were examined for their ability to lower blood pressure and reduce the pressor response to epinephrine and occlusion of the carotid arteries in healthy, normotensive, vagally denervated mongrel dogs, anesthetized with 35 mg./Kg. of pentobarbital sodium injected intravenously. Blood pressure was recorded directly from a femoral artery. In this study the chalcones were injected intravenously (femoral vein) in geometrically progressing doses beginning with 1 mg./Kg. Succeeding doses were injected after the blood pressure returned to pretreatment level or after an interval of twenty minutes if the preceding dose had no significant effect. This procedure continued until the death of the animal occurred. Epinephrine hydrochloride (0.25 to 0.5 ml. of a 1:20,000 solution, depending upon the sensitivity of the animal) was injected intravenously ten minutes after administration of each dose of the chalcone. Five minutes after the injection of epinephrine, the carotid sinus reflex was evoked by clamping both common carotid arteries for an interval of thirty seconds. The pressor responses following each procedure were compared with a series of uniform responses elicited prior to administration of the first dose of chalcone. The data obtained in this study are summarized in Table II.

With the exception of compound *A*, none of the chalcones examined produced a sustained (sixty minutes or more) reduction of blood pressure in sublethal concentrations. None of the animals survived the 32 mg./Kg. intravenous dose (representing a cumulative concentration of 63 mg./Kg.); in many cases death occurred rapidly after administration of the 16 mg./Kg. dose. Immediate post-mortem

[1] The chalcones employed in this study were generously supplied by Dr. Nathan Rubin, Department of Chemistry, Philadelphia College of Pharmacy and Science, and Dr. Albert Packman, Department of Organic Chemistry, National Drug Company Research Laboratories, Philadelphia, Pa.

TABLE I.—A NEW SERIES OF CHALCONE DERIVATIVES[a]



Chalcone

Code Letter	Group Substituted on Chalcone	Position on Chalcone	Anion
A	$(CH_3)_2NCH_2CH_2O$—	2	Citrate
C	$(C_2H_5)_2NCH_2CH_2O$—	2	Citrate
D	$(C_2H_5)_2NCH_2CH_2CH_2O$—	4	HCl
E	$(CH_3)_2NCH_2CH_2O$—	4	Citrate
F	$(CH_3)_2NCH_2CH_2CH_2O$—	4	HCl
G	$(C_2H_5)_2NCH_2CH_2O$—	4′	HCl
H	$(CH_3)_2NCH_2CH_2CH_2O$—	4′	HCl
I	$(C_2H_5)_2NCH_2CH_2O$—	2′	Citrate
J	$(CH_3)_2NCH_2CH_2O$—	4	HCl
K	$(CH_3)_2NCH_2CH_2O$—	4′	HCl
M	$(CH_3$—CH—$)_2NCH_2CH_2O$—	4	HCl
	$\quad\quad\quad

TABLE II.—EFFECTS OF A SERIES OF CHALCONES ON BLOOD PRESSURE, RESPONSE TO EPINEPHRINE, AND CAROTID ARTERY OCCLUSION[a]

		Dose in mg./Kg.[b]															
		1			2			4			8			16			32
Compd.	N[c]	BP[d]	Epi[e]	CSR[f]	BP	Epi	CSR	BP	Epi	CSR	BP	Epi	CSR	F[g]	BP	Epi	CSR	F
A	5	0	−32	−28	td[h]	−40	−44	32	−50	−58	61s[i]	−60	−75	2/5	72s	−67	−90	3/5
B	2	0	0	0	0	0	0	td	0	0	td	0	0	1/2	td	0	0	1/2
C	3	0	− 8	−15	td	−19	−25	19	−30	−56	41	−48	−90	2/3	50	−67	−90	1/3
D	2	0	0	0	td	0	0	td	0	−10	33	0	−47	1/2	24	−10	−90	1/2
E	2	0	0	0	td	+13	0	td	+21	0	td	+27	− 7	1/2	20	0	−90	1/2
F	2	0	0	0	0	0	−15	td	0	−25	td	0	−25	1/2	td	0	−20	1/2
G	2	0	− 9	0	td	0	0	td	0	+ 7	td	+25	0	1/2	td	+25	−37	1/2
H	2	0	0	0	0	0	0	td	0	0	td	0	−22	0	td	−15	−46	2/2
I	3	0	0	− 7	0	0	−10	td	0	−22	td	0	−20	2/3	td	+10	−20	1/3
J	2	0	0	0	0	0	+10	td	0	+45	td	−15	+65	0	td	−37	−12	2/2
K	2	0	0	0	0	0	−12	td	0	−17	td	0	−35	0	td	−11	−80	2/2
L	2	0	0	0	0	+14	0	0	+23	0	td	+22	−25	1/2	td	+10	0	1/2
M	3	0	0	−28	td	0	−40	td	0	−67	61	0	−90	2/3	90	0	−90	1/3
N	2	0	0	0	0	0	0	td	0	0	td	0	0	0	td	0	−40	2/2

[a] All experiments performed in pentobarbital sodium anesthetized, normotensive, vagally denervated mongrel dogs.
[b] All doses injected intravenously (femoral vein). Succeeding doses injected after BP returned to normal or after interval of twenty minutes if preceding dose had no significant effect.
[c] N = Number of animals used.
[d] BP = Mean decrease in blood pressure in mm. Hg (recorded from the femoral artery).
[e] Epi = Mean per cent change in response to intravenous epinephrine hydrochloride (0.25 to 0.5 ml. of 1:20,000 solution).
[f] CSR = Mean per cent change in response to occlusion of the carotid arteries for thirty seconds.
[g] F = Fatal (No. died/No. used). [h] td = Transient drop in blood pressure. [i] s = Sustained for 60 minutes or more.

TABLE III.—COMPARATIVE HYPOTENSIVE EFFECT OF COMPOUND *A* AND HYDRALAZINE IN THE HYPERTENSIVE RAT[a]

Mean Pretreatment Blood Pressure (Systolic), mm. Hg	Drug	Dose, mg./Kg.	Route of Administration	Mean Reduction in Blood Pressure, mm. Hg, Time in Hours after Administration of Drug				S. D., mm. Hg
				1	2	4	6	
230	Compound *A*	4	i. p.	24	25	11	0	±5
213	Hydralazine	0.4	i. p.	26	27	14	1	...
223	Compound *A*	8	i. p.	28	37	21	3	±3
222	Hydralazine	0.8	i. p.	70	77	50	3	...
215	Compound *A*	8	*per os*	10	16	7	1	±7
230	Hydralazine	0.8	*per os*	42	47	22	4	...
206	Compound *A*	16	*per os*	24	21	7	1	±8
208	Hydralazine	1.6	*per os*	58	58	25	8	...

[a] N = 10 in all groups.

did so within four hours after intraesophageal or intraperitoneal injection, or within thirty minutes after intravenous injection. In each case, death was preceded by salivation, ataxia, tremor, and clonic convulsions. Immediate postmortem examination revealed marked venous congestion and diastolic cardiac arrest. LD_{50} values and their limits of error were calculated according to a modification of the method of Epstein and Churchman (9).

The oral LD_{50} of compound *A* was found to be 603 mg./Kg. (95% Fiducial limits of 537 and 676 mg./Kg.); the intraperitoneal LD_{50} was determined to be 158 mg./Kg. (95% Fiducial limits of 148 and 170 mg./Kg.); and the intravenous LD_{50} was calculated to be 40.8 mg./Kg. (95% Fiducial limits of 37.2 and 44.7 mg./Kg.) in mice.

Administration of equivalent doses of compound *A* to female Sherman rats (Scott Farms) weighing from 160 to 175 Gm. indicated the LD_{50} values to be within the ranges noted for mice. The intravenous LD_{50} of compound *A* obtained in mice closely corresponds to the intravenous doses which produced death in anesthetized dogs during the determination of the hypotensive activity of this molecule.

Reduction of Epinephrine Toxicity in Mice.—The degree and duration of protection provided by compound *A* against the lethal effect of epinephrine in mice was determined according to the method of Loew and Micetich (10) with minor modification. The doses of compound *A* employed were 39.5 and 79 mg./Kg. (representing $^1/_4$ and $^1/_2$ of the intraperitoneal LD_{50}, respectively), injected intraperitoneally into various groups each composed of 20 mice. These doses of compound *A* were compared to 10 mg./Kg. of tolazoline (Priscoline) hydrochloride injected intraperitoneally. At varying time intervals (15, 30, 60, and 120 minutes) after administration of either saline, compound *A* or tolazoline, the mice were injected intraperitoneally with a standard dose of 14.4 mg./Kg. of epinephrine hydrochloride. This concentration of epinephrine sufficed to kill an average of 85% of the unprotected (saline injected) control animals as indicated by the dotted line in Fig. 1.

Administration of both $^1/_4$ and $^1/_2$ of the intraperitoneal LD_{50} of compound *A* reduced the lethal effects of epinephrine at the 15, 30, and 60-minute test periods. This activity was dissipated in two hours and the mortality returned to the control

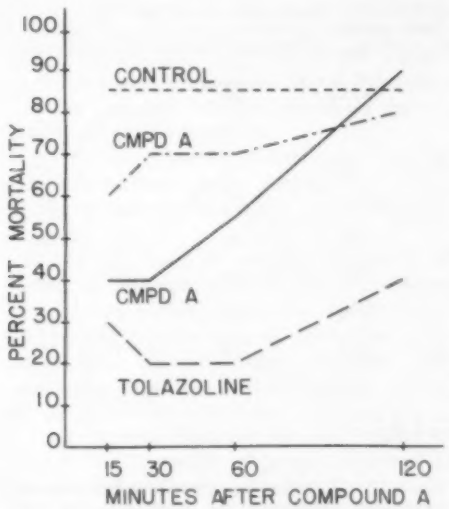


Fig. 1.—Reduction of epinephrine toxicity in mice. Saline control -----; Compound *A* 39.5 mg./Kg., i.p., ——; compound *A* 79 mg./Kg., i.p.,——; Tolazoline 10 mg./Kg., i.p., — —. Each group of 20 mice received epinephrine HCl, 14.4 mg./Kg., i. p., at the time interval indicated after administration of the test solution.

level. A greater degree and duration of protection against the toxic effects of epinephrine was provided by a significantly smaller dose (10 mg./Kg.) of tolazoline.

The reduction of epinephrine toxicity in mice with a given compound does not necessarily indicate adrenergic blocking activity (10). Other experiments have demonstrated that compound *A* possesses marked cardioinhibitory and vasodilator activity, both effects which might account for its ability to decrease the lethal action of epinephrine.

Prolongation of Sleeping Time in Mice.—During the evaluation of the hypotensive activity of compound *A* in pentobarbital sodium anesthetized dogs, it was consistently noted that the animals remained unconscious for protracted periods without supplementation of the original dose of anesthetic. On this basis it was considered desirable to investigate the effect of this chalcone on the duration of barbiturate induced sleeping time in mice.

Compound *A* was injected intraperitoneally into varying groups of mice[4] in doses of 39.5 and 79 mg./Kg. (representing $^1/_4$ and $^1/_2$ of the intraperitoneal LD_{50}, respectively) ten minutes prior to intraperitoneal administration of either saline (control), 45 mg./Kg. of pentobarbital sodium or 112 mg./Kg. of hexobarbital sodium. The interval between administration of the barbiturate and reappearance of the "righting reflex" (animal turns spontaneously over into normal position but may continue to sleep) was recorded as the "sleeping time" (11).

Pretreatment with doses of compound *A* (39.5 and 79 mg./Kg.) which produced only mild ataxia, markedly prolonged the duration of hypnosis in mice induced by either pentobarbital sodium or hexobarbital sodium (Table IV). "Sleeping time" following administration of 45 mg./Kg. of pentobarbital sodium was increased by a factor of 5.8 by prior injection of 79 mg./Kg. of compound *A* without any mortality in the group. The hypnotic effect of hexobarbital sodium, in the dose employed, was also prolonged but to a lesser extent.

This experiment was repeated using the same materials as outlined above, but the procedure was modified by administration of compound *A* ten minutes after injection of the barbiturates. The prolongation of "sleeping time" following post-treatment with compound *A* was considerably less than that obtained by pretreatment with comparable doses of the chalcone (results not reported). This difference may be largely attributed to the relatively short duration of action of the hydroxychalcones.

At this time we cannot account for the mechanism by which compound *A* prolongs the barbiturate hypnosis. Preliminary investigation in this direction indicates a reduction in the rate of barbiturate excretion.

Antihistaminic Activity.—The techniques employed and the results obtained in the evaluation of the antihistaminic and antispasmodic activity of this new series of chalcone derivatives have been previously described in detail by Rossi and Avellino (12), therefore this phase of the investigation will be only briefly discussed in the present report.

The ability of each chalcone in the series to inhibit histamine, acetylcholine, and barium chloride induced spasms was compared with diphenhydramine (Benadryl), atropine, and papaverine on isolated guinea pig ileum according to a modification of the Magnus method. On the basis of the techniques employed, compounds *A*, *C*, and *D* exhibited the greatest antispasmodic activity *in vitro*. The results obtained with these three chalcones on isolated gut are summarized in Table V.

The degree of inhibition of the characteristic depressor response to intravenous histamine in anesthetized cats obtained with various doses of the three most effective chalcones is compared with diphenhydramine in Table VI. These data indicate compounds *A*, *C*, and *D* to be significantly less active than diphenhydramine *in vivo*. It is generally recognized, however, that this procedure frequently does not provide an adequate measure of the potency differences between antihistaminic compounds.

From the data presented in Tables V and VI, it is evident that both the antihistaminic potency and specificity of diphenhydramine are considerably greater than any of the compounds investigated. It may therefore be concluded that this group of chalcones is devoid of significant antihistaminic activity.

DISCUSSION

A new series of chalcone derivatives synthesized as potential hypotensive agents have been evaluated for blood pressure reducing activity in the anesthetized normotensive dog. On the basis of this preliminary investigation, the most effective member of this series, 2-(2-dimethylaminoethoxy) chalcone

[4] Described in the section on acute toxicity.

TABLE IV.—EFFECT OF COMPOUND *A* ON PENTOBARBITAL SODIUM AND HEXOBARBITAL SODIUM SLEEPING TIME IN MICE

First Injection	mg./Kg. I. P.	Second Injection 10 Minutes after First Injection	mg./Kg. I. P.	Mean Sleeping Time, Min.	Ratio	N[a]
Compound *A*	39.5[b]	Saline	...	0	...	40
Compound *A*	79.0[c]	Saline	...	0	...	40
Saline	...	Pentobarbital Sodium	45	17	1.0	40
Compound *A*	39.5	Pentobarbital Sodium	45	37	2.2	20
Compound *A*	79.0	Pentobarbital Sodium	45	98	5.8	20
Saline	...	Hexobarbital Sodium	112	29	1.0	40
Compound *A*	39.5	Hexobarbital Sodium	112	51	1.7	20
Compound *A*	79.0	Hexobarbital Sodium	112	70	2.4	20

[a] N = number of animals used.
[b] 39.5 mg./Kg. is equivalent to 1/4 of the i. p. LD_{50} of compound *A* in mice.
[c] 79.0 mg./Kg. is equivalent to 1/2 of the i. p. LD_{50} of compound *A* in mice.

TABLE V.—AVERAGE PER CENT INHIBITION OF THE CONTRACTILE RESPONSE TO HISTAMINE, ACETYLCHOLINE, AND BARIUM CHLORIDE IN THE ISOLATED GUINEA PIG ILEUM BY GRADED DOSES OF THE MOST ACTIVE CHALCONES

Histamine, 10 mcg./100 ml.

Compound	Concn. of Antagonist in mg./100 ml. 0.0001	0.001	0.01	0.1
A	0[a]	0	0	10
C	0	0	8	18
D	0	0	0	14
Diphenhydramine	0	73	85	...

Acetylcholine, 100 mcg./100 ml.

Compound	Concn. of Antagonist in mg./100 ml. 0.01	0.1	1.0
A	0	0	54
C	0	58	97
D	0	60	96
Diphenhydramine	0	70	100
Atropine	100	...	...

Barium Chloride, 500 mcg./100 ml.

Compound	Concn. of Antagonist in mg./100 ml. 0.5	5.0	50.0
A	66	97	100
C	50	100	100
D	66	98	100
Diphenhydramine	56	100	100
Papaverine	10	95	100

[a] Each figure represents the average of determinations made on three isolated muscle strips.

citrate (designated as compound *A*) was subjected to further study of depressor activity in the dog and in the unanesthetized hypertensive rat. Compound *A*, administered orally and parenterally, provided moderate reduction of blood pressure but for a period of time insufficient to suggest possible clinical usefulness. The mechanism of compound *A* in reducing blood pressure has not been fully elucidated, but appears to be the result of direct smooth muscle relaxation in addition to incomplete adrenergic blockade.

The ability of the fourteen new chalcones to inhibit histamine, acetylcholine, and barium chloride induced spasm was compared with diphenhydramine, atropine, and papaverine on isolated guinea pig ileum. The three chalcones (compounds *A*, *C*, and *D*) found most effective *in vitro* were also examined for their ability to reduce the histamine depressor response in the anesthetized cat. Although antihistaminic and antispasmodic activity was manifested by certain members of this series, the potency and duration of activity was significantly less than that displayed by presently available compounds in these categories.

TABLE VI.—AVERAGE PER CENT INHIBITION OF THE DEPRESSOR RESPONSE TO HISTAMINE IN THE ANESTHETIZED CAT BY GRADED DOSES OF THE MOST ACTIVE CHALCONES

Histamine, 1 mcg./Kg. Intravenously

Compound	Dose of Antagonist in mg./Kg. Intravenously 1	2	3	4
A	0[a]	0	0	10
C	0	11	23	38
D	0	0	0	0
Diphenhydramine	...	...	...	68

[a] Each figure represents the average of determinations made on two or more animals.

The series of hydroxychalcones which are the subject of this report represented an interesting theoretical approach to the synthesis of hypotensive agents. The derivatives examined shared the major disadvantages of low potency, lack of specificity, and short duration of action. Synthesis of additional members of this series may provide chalcones with greater hypotensive or antihistaminic effectiveness.

SUMMARY

1. A series of fourteen new chalcone derivatives were evaluated with regard to their hypotensive potency and ability to inhibit the pressor responses to epinephrine and occlusion of the carotid arteries in anesthetized normotensive dogs.

2. The blood pressure reducing activity of the most effective member of the series, 2-(2-dimethylaminoethoxy) chalcone citrate (compound A), was compared with hydralazine in the unanesthetized hypertensive rat.

3. The acute toxicity of compound *A* and its influence on epinephrine toxicity and barbiturate induced "sleeping time" were determined in mice.

4. On the basis of structural similarity to certain antihistamines, the activity of this series of chalcones against histamine, acetylcholine, and barium chloride induced spasms was compared with diphenhydramine, atropine, and papaverine on isolated guinea pig ileum. Three of the most effective chalcones (compounds *A*, *C*, and *D*) were examined for their ability to inhibit the histamine depressor response in anesthetized cats.

5. The chalcones investigated in this study exhibited relatively low specificity, low potency, and transient duration of action. Synthesis of additional chalcones, with consideration given to the activities reported, is recommended.

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Anticonvulsant Drug Combinations: Diphenylhydantoin Combined with Other Antiepileptics*

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Methods are presented for the study of drug combinations in which one component is active by the anticonvulsant test and both components are active by the neurotoxicity test. The effects of Dilantin on anti-Metrazol activity and neurotoxicity of five clinically useful antiepileptics were determined in mice and protective indices calculated. The anti-Metrazol activity of two combinations of Dilantin-Phenurone was significantly greater than that of Phenurone alone. The minimal neurotoxic doses of all combinations of Dilantin-Tridione and Dilantin-Mesantoin were significantly larger than those calculated on the basis of similar joint action of toxicities. The margin of safety of one combination of Dilantin-Mesantoin was significantly increased over that of the active component alone.

A PREVIOUS PUBLICATION (1) described a method for the laboratory evaluation of combinations of two antiepileptic agents, both of which are active by the assay procedure employed; the anticonvulsant activity of various combinations of diphenylhydantoin (Dilantin) and phenobarbital were determined by the maximal electroshock seizure test. These studies have now been extended to anticonvulsant drug combinations in which only one component of the mixture is active by the test employed. Dilantin, a compound ineffective against pentylenetetrazol (Metrazol)-induced seizures (2, 3), was combined with agents known to be effective against this convulsant, and the anti-Metrazol activity of the mixtures was then determined. In addition, the minimal neurotoxic dose for each combination was measured. The results obtained provide the basis for this report.

METHODS

Assay Procedures.—Phenobarbital sodium, phenacemide (Phenurone[1]), trimethadione (Tridione[1]), methylphenylethylhydantoin (Mesan-

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[1] Supplied by Abbott Laboratories.

toin[2]), and mephobarbital (Mebaral[3]), alone and in combination with diphenylhydantoin (Dilantin[4]) sodium, were tested in mice for ability to prevent pentylenetetrazol (Metrazol[5])-induced seizures and for neurotoxicity. The quantity of each active drug which protected 50% of mice from Metrazol (ED_{50}) was combined with 3, 6, and 12 mg./Kg. of the inactive drug (Dilantin); in two experiments Phenurone and Tridione were combined with 24 and 30 mg./Kg. of Dilantin, respectively. Each combination was evaluated as a new drug. All drugs and all drug combinations were administered orally in 10% acacia solution. Male albino mice (Carworth Farms, CF #1 strain), 17 to 37 Gm. wt. were used as experimental animals; they were allowed free access to food (Purina Laboratory Chow) and water except during the actual test period. Anticonvulsant activity was determined by the Metrazol seizure threshold (Met.) test (85 mg./Kg. administered subcutaneously as a 0.5% aqueous solution) and neurotoxicity was determined on the basis of signs of minimal overt neurological deficit. The details of the Met. test and the end points employed for the determination of acute neurotoxicity in mice have been described elsewhere (4). All tests were performed at the time of peak activity of the active component (see footnotes, Table I).

In the determination of anticonvulsant activity and neurotoxicity, groups of 8 to 12 mice were tested with various doses of drug or drug combination until at least three points were established between the limits of complete protection or toxicity and no protection or toxicity. The results obtained were plotted on logarithmic probability paper and regression lines fitted to the plotted points by the method of maximum likelihood. The dose of drug or drug combination producing the desired end point (protection or neurotoxicity) in 50% of animals (ED_{50} or TD_{50}) was computed. The regression lines for anti-Metrazol activity and neurotoxicity of each drug and drug combination were tested for parallelism for the Chi-square method described by Finney (5), and protective indices (TD_{50}/ED_{50}) were calculated. In those instances where fiducial limits were desired, a method described by Finney (5) was utilized.

Statistical Analysis.—The values obtained by the Met. test were analyzed for potentiation or antagonism of anticonvulsant activity. A significant change in anti-Metrazol activity was considered to have occurred when the 95% fiducial limits of the active component in the combination were outside those of the active component alone. When significantly less of the active drug was required for 50% protection, the result was considered to represent potentiation; conversely, when significantly more of the active drug was required, the result was considered to represent antagonism. Since all drugs and combinations used in these studies exhibited toxicity in appropriate doses, the neurotoxicity values obtained were statistically analyzed by the method described by Finney (5) for any departure from the prediction of similar joint action.

[2] Supplied by Sandoz Chemical Works, Inc.
[3] Supplied by Sterling-Winthrop Research Institute.
[4] Supplied by Parke-Davis Company.
[5] Supplied by Bilhuber-Knoll Corporation.

RESULTS

The data obtained are summarized in Table I. The ED_{50}'s calculated by the probit method of analysis are listed under the column marked anti-Metrazol activity. Under the general title neurotoxicity are listed the calculated TD_{50}'s, observed TD_{50}'s, toxicity ratios, mean probit differences, and P values for the mean probit differences. The calculated TD_{50}'s were mathematically derived by the probit method of Finney (5) for similar joint action, whereas the observed TD_{50}'s were determined experimentally. The toxicity ratios were derived by dividing the calculated toxicity value by the observed toxicity value; numbers below 1.00 indicate an effect less than that calculated; numbers above 1.00 indicate an effect greater than that calculated. The mean probit difference is the constant vertical difference between the two parallel probit regression lines; it measures only the difference in effect of equal doses; it does not compare the magnitudes of equally effective doses. Comparison of the mean probit difference with its standard error provides a test for the significance of the departure from the prediction of simple addition on the basis of similar joint action; positive values of the mean probit difference indicate that the observed toxicity is greater than predicted; negative values, less than predicted. The P value denotes the significance of the departure. The protective index (observed TD_{50} divided by the ED_{50}) is shown in the last column. Because the toxicity regression lines for Phenurone and Phenurone-Dilantin combinations were not parallel, it was not possible to analyze the data by the method of probit analysis; therefore, these data were analyzed by the same method as used for anticonvulsant activity.

A study of Table I reveals that, except for two combinations with Phenurone, Dilantin had no significant effect on the anti-Metrazol activity of the five drugs tested. In the two exceptions, the amount of Phenurone required to protect 50% of animals was significantly reduced by the addition of small amounts of Dilantin. For example, the ED_{50} and 95% fiducial limits of the combination composed of 16.7% Dilantin and 83.3% Phenurone were 74.1 (56.0–98.2) mg./Kg., whereas for Phenurone alone they were 119.7 (111.2–129.5) mg./Kg. Thus, the anti-Metrazol activity of this particular combination was increased by approximately 38% over that of Phenurone alone.

The neurotoxicity of Mebaral, Mesantoin, and Tridione was decreased below that expected (based on the prediction of similar joint action) by the addition of small amounts of Dilantin, whereas the neurotoxicity of phenobarbital was not altered by such addition. Although no quantitative determination could be made of the effect of Dilantin on the neurotoxicity of Phenurone, the fact that the margins of safety (protective indices) were not significantly changed suggests that the increase in neurotoxicity merely paralleled the increase in anti-Metrazol activity.

Except for one combination (Dilantin 6.5%-Mesantoin 93.5%), there was no significant alteration in the protective indices of the seventeen combinations tested. The protective index and 95% fiducial limits for this particular Dilantin-

TABLE I.—EFFECT OF DILANTIN SODIUM ON THE ANTI-METRAZOL ACTIVITY, NEUROTOXICITY, AND PROTECTIVE INDEX OF FIVE ANTIEPILEPTIC DRUGS

Drug or Drug Combination: Active Component	% [a]	Anti-Metrazol Activity, ED_{50}, Active Component, mg./Kg.	Neurotoxicity: Calculated TD_{50}, mg./Kg.	Neurotoxicity: Observed TD_{50}, mg./Kg.	Neurotoxicity: Toxicity Ratio	Neurotoxicity: Mean Probit Difference ± S. E.	Neurotoxicity: *P*, for Mean Probit Difference	Protective Index
Phenobarbital Sodium	100.0	20.3 (18.9–21.8)	...	78.6	...	...	...	3.87 (3.40–4.42)
	87.2	15.9 (13.1–19.3)	80.8	75.7	1.07	+0.42 ± 0.41	0.1	4.76
	77.4	19.0 (15.9–21.8)	82.6	84.1	0.98	−0.12 ± 0.41	0.7	4.43
	63.0	17.6 (15.7–19.7)	85.0	84.4	1.81	+0.06 ± 0.40	0.7	4.80 (3.90–5.90)
Mebaral	100.0	19.8 (16.8–23.3)	...	64.1	...	...	...	3.24 (2.57–4.07)
	87.5	18.0 (14.8–22.0)	66.6	83.2	0.80	−0.73 ± 0.39	0.05	4.62 (3.39–6.31)
	77.8	16.0 (13.5–19.0)	68.1	83.2	0.82	−0.66 ± 0.38	0.05	5.19 (4.05–6.65)
	63.6	19.1 (17.3–21.1)	72.2	87.9	0.82	−0.64 ± 0.32	0.05	4.62 (3.25–6.59)
Mesantoin	100.0	42.8 (35.7–51.2)	...	106.0	...	...	...	2.48 (1.96–3.13)
	93.5	34.3 (28.8–40.8)	106.3	143.9	0.74	−1.90 ± 0.48	0.001	4.20 (3.43–5.14)
	87.8	45.9 (41.3–51.1)	106.4	142.7	0.75	−1.83 ± 0.43	0.001	3.11
	78.3	51.6 (41.0–65.0)	106.7	148.5	0.72	−2.06 ± 0.43	0.001	2.89
Tridione	100.0	371.6 (342.8–402.7)	...	774.1	...	...	...	2.08 (1.91–2.33)
	99.2	363.4 (330.4–399.1)	733.0	992.6	0.74	−2.03 ± 0.36	0.001	2.73
	98.5	381.6 (261.3–558.4)	696.5	1070.0	0.65	−2.88 ± 0.32	0.001	2.80 (1.91–4.11)
	96.9	372.3 (314.0–441.5)	634.3	1062.8	0.60	−3.46 ± 0.29	0.001	2.85 (2.37–3.43)
	92.7	331.8 (171.8–641.3)	507.0	858.6	0.59	−3.53 ± 0.34	0.001	2.59
Phenurone	100.0	119.7 (111.2–129.5)	... [b]	372.6 [c] (344.4–402.7)	...	...	...	3.11 (2.61–3.70)
	97.6	100.6 (90.4–112.3)	...	372.1 (342.0–405.5)	...	...	...	3.70
	95.2	102.1 (83.8–124.5)	...	313.3 (270.4–363.1)	...	...	...	3.08
	90.9	94.2 (83.8–105.9)	...	352.2 (311.9–398.1)	...	...	...	3.74
	83.3	74.1 (56.0–98.2)	...	265.2 (226.7–311.2)	...	...	...	3.59
Dilantin Sodium	100.0	... [d]	...	... [e]	...	...	...	...

[a] Made up to 100% with Dilantin sodium. [b] See text for discussion.
[c] 95% confidence limits. [d] Inactive by this test.
[e] Control TD_{50}'s for Dilantin Sodium were determined at the following times which represent the times of peak effect for the orally administered active compound given in parentheses: 3.0 hr. (phenobarbital sodium) 99.6 mg./Kg.; 2.5 hr. (Mebaral) 93.3 mg./Kg.; 1.5 hr. (Mesantoin) 109.5 mg./Kg.; 2.0 hr. (Tridione) 94.4 mg./Kg.; and 1.0 hr. (Phenurone) 120 mg./Kg.

Mesantoin combination were 4.20 (3.43–5.14), whereas the comparable values for Mesantoin alone were 2.48 (1.96–3.13); this represents approximately a 70% improvement in margin of safety over that of Mesantoin alone.

DISCUSSION

The difficulties encountered in the quantitative analysis of the effects of drug combinations composed of two or more components, both of which are active by the test employed, have been discussed in previous publications from our laboratories (1, 6). Fortunately, when only one component of the mixture is active by the assay procedure employed, the quantitative analysis of the combined drug effects is less complex. Indeed, such combinations can be readily assayed and the results directly compared with similar data obtained with the active compound alone. Thus, in the present studies, a significant alteration in the anti-Metrazol activity of combinations of the active drug and Dilantin (a drug inactive against Metrazol) was considered to indicate potentiation or antagonism of drug action, depending on the direction of the alteration.

It is noteworthy that the anti-Metrazol activity of Phenurone increased as the dose of Dilantin increased. This suggests that the potentiating effects of Dilantin on the anti-Metrazol activity of Phenurone was dose-dependent, and indicates that even higher doses of Dilantin should be investigated. However, since there was no significant improvement in the protective index after a four-fold increase in the dose of Dilantin and because of the stimulant effects known to occur with high doses of Dilantin, it was not considered advisable to extend the Dilantin dose range.

When compared on the basis of neurotoxicities, it may be seen that the results obtained with combinations of Dilantin-phenobarbital and Dilantin-Mebaral agree with the prediction of similar joint action. According to the previously described interpretations (1), the combinations composed of Mesantoin and Tridione in combination with Dilantin exhibit antagonism of toxicity. Although the Dilantin-Phenurone toxicity data could not be evaluated by the probit method because the regression lines were not parallel, the data indicate a trend toward increased toxicity which paralleled the increase in anti-Metrazol activity.

As previously suggested by Loewe (6), it is more important to study the ratio between the intensities of various effects of the same combination, i. e., to know whether these ratios (margins of safety or protective indices) assume a large or smaller value for the combinations than for the components. The combination composed of Dilantin 6.5%-Mesantoin 93.5% exhibited a protective index which was approximately 70% higher than that for Mesantoin alone.

Since a major objective of this study was to present the experimental design and the statistical analysis for evaluating drug combinations in which only one component of the mixture is active by the test employed, no concerted effort was made to cover the entire spectrum of activity of each component or combination. Hence, speculation on the laboratory and clinical implications of these data is not justified. Nevertheless, the results obtained with combinations of Dilantin-Phenurone and Dilantin-Mesantoin are sufficiently promising to warrant their further study.

SUMMARY

Methods are presented for the study of anticonvulsant drug combinations in which only one component is active by the assay test employed and both components are active by the neurotoxicity test. To illustrate this procedure, small amounts of Dilantin, an agent ineffective against Metrazol-induced seizures, were combined with known effective doses of phenobarbital sodium, Mebaral, Mesantoin, Tridione, and Phenurone, and the anti-Metrazol activity and neurotoxicity of each combination were determined; in addition, the protective indices were calculated. The anticonvulsant data obtained were analyzed for evidence of antagonism or potentiation of anti-Metrazol activity, whereas the toxicity data were analyzed for any departure from the prediction of similar joint action.

The results obtained may be summarized as follows:

1. Dilantin significantly increased the anti-Metrazol activity of the two combinations which contained 90.9 and 83.3% Phenurone, by approximately 27 and 38%, respectively; but Dilantin had no effect on the activity of phenobarbital, Mebaral, Tridione, or Mesantoin.

2. The neurotoxicities were significantly decreased in all combinations of Dilantin with Tridione, Mesantoin, and Mebaral, unchanged in all combinations of Dilantin with phenobarbital, and apparently increased in all combinations of Dilantin with Phenurone although nonparallelism of regression lines precluded the quantitative evaluation of this latter set of combinations.

3. One of the seventeen combinations tested, Dilantin 6.5% with Mesantoin 93.5%, exhibited a 70% increase in margin of safety.

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Study of Stability of Sulfadiazine Sodium Injection IV.*

The Determination of Oxygen Uptake of Sulfadiazine Sodium with Antioxidants in Solution

By CHARLES J. SWARTZ† and JOHN AUTIAN‡

The addition of certain antioxidants to ampuls of sulfadiazine sodium solution prepared in a nitrogen atmosphere retards the formation of color. The efficiency of various antioxidants to inhibit the development of color was investigated by determining the ability of these agents to utilize available oxygen in sulfadiazine sodium solutions at various hydrogen ion concentrations. Quantitative estimation of oxygen uptake was achieved by the utilization of a Warburg Constant Volume Respirometer. Ascorbic acid, *p*-aminophenol, hydroquinone, sodium formaldehyde sulfoxylate, sodium sulfite, and *tert*-butyl hydroquinone were the antioxidants evaluated. Oxygen consumption data were correlated with actual formation of color in ampuls autoclaved for prolonged periods.

It was observed in earlier studies (1, 2) that the addition of antioxidants to ampuls of sulfadiazine sodium, sealed under an atmosphere of nitrogen, prevented or delayed the formation of color in these solutions. The antioxidants varied in their ability to hinder this coloration. The solvent, polyethylene glycol, also seemed to have some activity in retarding color formation (2).

It was, therefore, decided to measure the amount of oxygen consumed by solutions of sulfadiazine sodium with antioxidants employing a Warburg apparatus. These values were then correlated with the ability of the various agents to prevent color formation in ampuls subjected to prolonged periods of autoclaving.

EXPERIMENTAL

Determination of Oxygen Consumption.—Oxygen consumption was determined with the aid of a Warburg apparatus utilizing standard procedures as described by Umbreit, *et al.* (3). Single side-arm Warburg flasks of approximately 15-ml. volume were employed in all tests. Fluid volume was kept at 4.0 ml. throughout.

The experimental flasks contained 3.0 ml. of 25.0% sulfadiazine sodium solution buffered at pH 9.75 and 1.0 ml. of a 1.0% solution of the antioxidant under study. Control flasks were prepared containing 3.0 ml. of distilled water and 1.0 ml. of antioxidant. Flasks containing 3.0 ml. of a buffer solution of pH 9.75 and 1.0 ml. of antioxidant were also prepared.

Oxygen consumption was determined at a temperature of 50.0 ± 0.02° in an atmosphere of air. Preliminary tests indicated that higher oxygen tensions were not suitable for these studies since the reaction proceeded too rapidly. After a fifteen minute equilibration period, readings were recorded at ten minute intervals for three hours. From the readings obtained, the corresponding volume of oxygen consumed by the contents of each flask was calculated in the usual fashion.

Antioxidants which were evaluated in these experiments included: sodium sulfite, sodium formaldehyde sulfoxylate, ascorbic acid, hydroquinone, *tert*-butyl hydroquinone, and *p*-aminophenol. The following combinations of agents were also tested: sodium sulfite and ascorbic acid, sodium formaldehyde sulfoxylate and ascorbic acid, and citric acid with each of the above mentioned three.

Preparation of Ampuls for Autoclaving.—Twenty-milliliter clear glass ampuls Type 2 were used throughout the study. Aqueous solutions of sulfadiazine sodium, buffered at pH 9.75, were prepared containing the antioxidants (in 0.05% concentration) listed in Table 1. The use of hydroquinone, *t*-butyl hydroquinone and *p*-aminophenol was not feasible in this phase of the study as the oxidized forms of the above three agents are highly colored. Consequently, these antioxidants could not be adequately evaluated.

Fifteen milliliters of solution was placed in each ampul and triplicate determinations were made on each series. The ampuls were placed in an electric steam pressure sterilizer (pressure cooker type) after being sealed in the presence of air. Per cent transmittance readings were recorded for these freshly prepared samples utilizing a Beckman Model C colorimeter with a prefocused tungsten lamp and a Corning 4010 green filter. The pressure was regulated to 15–16 p.s.i. A period of twelve hours was found to be adequate to allow the reactions to occur after which the ampuls were removed from the sterilizer. Per cent transmittance of the autoclaved solutions was measured after the contents of the ampuls had returned to room temperature.

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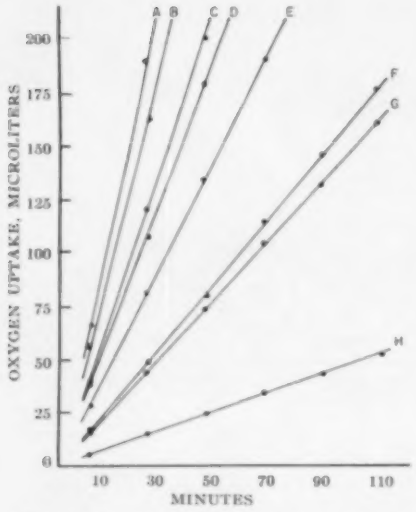

Fig. 1.—Oxygen uptake data for solutions of sulfadiazine sodium 18.75% with various antioxidants. Concentration of each antioxidant-0.25%. A—Sodium Sulfite, B—Hydroquinone, C—*p*-Amino phenol, D—Sodium Formaldehyde Sulfoxylate with Ascorbic Acid, E—Ascorbic Acid, F—Sodium Sulfite with Ascorbic Acid, G—*t*-Butyl Hydroquinone, H—Sodium Formaldehyde Sulfoxylate.

RESULTS AND DISCUSSION

The oxygen uptake for each solution was plotted against time in minutes and appears in Fig. 1. It will be noticed that all the curves are smooth in the range studied and that the slope of the lines may be used to indicate the protective effect that these antioxidants have upon sulfadiazine sodium in aqueous solution. A review of the slopes points to the fact that sodium sulfite (A) had the greatest antioxidant effect in comparison to the other agents used in this evaluation. Hydroquinone (B), *p*-amino phenol (C), sodium formaldehyde sulfoxylate with ascorbic acid (D), ascorbic acid (E), sodium sulfite with ascorbic acid (F), and *tert*-butyl hydroquinone (G) each had a slower rate of oxygen uptake while sodium formaldehyde sulfoxylate (H) had the slowest. Since a number of these antioxidants, themselves, upon oxidation produced coloration, they were removed from further consideration. An antioxidant synergist, citric acid, although reported to enhance the action of numerous antioxidants (4), proved ineffectual in the systems studied. It was felt that this experiment would not serve a very useful purpose unless the "oxygen uptake" could be related to the practical aspects of the problem. With this point in view, Fig. 2 was prepared. A quantitative relationship could not be observed, but a satisfactory qualitative comparison was possible. For example, when a system (Ampul A in Fig. 2) composed of sulfadiazine sodium and sodium sulfite in an aqueous medium was autoclaved for twelve hours, it produced less coloration than the other systems as measured photometrically. This same system in the Warburg experiments consumed 100 microliters of oxygen in the shortest time period (approximately ten minutes). In Ampul B containing two antioxidants (sodium formaldehyde sulfoxylate and ascorbic acid), a more intense color was produced than in Ampul A and the time for oxygen consumption of 100 microliters was increased. This type of relationship was progressively increased (more color and greater time period for uptake of oxygen) with Ampuls C, D, and E respectively. The control solution (Ampul F), sulfadiazine sodium alone, produced the most coloration. Since the oxygen utilization of sulfadiazine is infinitely slow, it could not be measured by the techniques employed.

Figure 2 also demonstrates the synergistic effect that ascorbic acid had upon sodium formaldehyde sulfoxylate. The per cent transmittance of sodium sulfadiazine in the presence of sodium formaldehyde sulfoxylate was 76% and this rose to 94% when ascorbic acid was included in the system. All reactions containing the buffered sulfadiazine sodium solutions showed increased activity over con-

TABLE I.—PER CENT TRANSMITTANCE OF SULFADIAZINE SODIUM WITH VARIOUS ANTIOXIDANTS AFTER TWELVE HOURS IN AUTOCLAVE AT 15 P.S.I.

Ampul[a]	Contents		Transmittance, % Initial	End
A	Sulfadiazine sodium	18.75%		
	Sodium sulfite	0.05%	100	95
B	Sulfadiazine sodium	18.57%		
	Sodium formaldehyde sulfoxylate	0.025%		
	Ascorbic acid	0.025%	100	94
C	Sulfadiazine sodium	18.75%		
	Ascorbic acid	0.05%	100	89
D	Sulfadiazine sodium	18.75%		
	Sodium sulfite	0.025%		
	Ascorbic acid	0.025%	100	83
E	Sulfadiazine sodium	18.75%		
	Sodium formaldehyde sulfoxylate	0.05%	100	76
F	Sulfadiazine sodium	18.75%	100	62

[a] Letters refer to samples in Fig. 2.

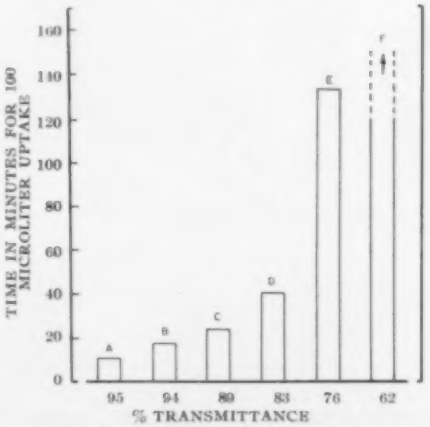

Fig. 2.—Graph illustrating relation between rate of oxygen uptake *vs.* formation of color in ampuls of sulfadiazine sodium (18.75%) with antioxidants. F = control sulfadiazine sodium.

trol runs. This action cannot be ascribed to ionic strength or pH factors as experiments investigating each of these yielded negative results. Unreported studies also disclosed that if the concentration of antioxidant was increased, no coloration appeared, after twelve hours of autoclaving, in any of the ampuls.

SUMMARY

Oxygen uptake studies utilizing a Warburg Constant Volume Respirometer were conducted on solutions of sulfadiazine sodium with the addition of various antioxidant agents.

The rate of oxygen consumption in these solutions was shown to be directly related to the ability of the antioxidant to deter color formation in ampuls of sulfadiazine sodium solutions. The tests showed sodium sulfite to have the greatest activity in the system under the conditions of study. A combination of sodium formaldehyde sulfoxylate and ascorbic acid was shown to be almost equally effective.

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The Adsorption of Enterobacterial Endotoxins by Activated Attapulgite*

By ERWIN NETER† and EUGENE A. GORZYNSKI‡

The effect of activated attapulgite upon endotoxins (lipopolysaccharides) of enterobacterial pathogens (enteropathogenic *Escherichia coli* and *Shigella sonnei*) was studied by means of the enterobacterial hemolysis and hemolysis inhibition tests and *in vivo* experiments in mice. It was found that activated attapulgite adsorbs the endotoxins as demonstrated by inhibition of enterobacterial hemolysis, elimination of the specific inhibitory effect of the antigens in hemolysis inhibition tests, and by the abolishment of toxicity in mice. The significance of the findings is discussed with particular reference to their bearing upon the clinical use of activated attapulgite in localized intestinal infections and other syndromes associated with these endotoxins.

RECENT STUDIES by Barr and Arnista (1) have revealed that activated attapulgite adsorbs diphtheria toxin and is approximately five times as effective as kaolin. The question arises as to whether this material also adsorbs endotoxins of enteric bacteria. To this end, the present study was carried out, utilizing the enterobacterial hemolysis and hemolysis inhibition tests and *in vivo* toxicity determinations as indicator systems.

The endotoxins of enteric bacteria, such as *Escherichia* (*E.*) *coli*, salmonellae, and shigellae, are composed of polysaccharide, lipid, and protein. These complexes, like the lipopolysaccharide fractions thereof, possess toxicity in mice and other animals, are extraordinarily pyrogenic, profoundly affect natural resistance to unrelated challenge infections, and act as antigens (haptens) in various serologic procedures. It is the polysaccharide that determines the antigenic characteristics of the complex. Recent studies suggest that toxicity and pyrogenicity are associated with the lipid component (2, 3). Studies from our laboratories have revealed that these enterobacterial lipopolysaccharides readily become attached to red blood cells and thus endow the latter with a newly acquired serologic specificity (4–6). As a result, these modified erythrocytes are specifically agglutinated in the presence of homologous antibodies, and hemolysis occurs upon the addition of antibodies and guinea pig complement, notably when sheep red blood cells are employed. These hemagglutination and hemolysis reactions have proved to be considerably more sensitive for the demonstration of homologous antibodies than the conventional bacterial agglutination procedures. Specific hemolysis is inhibited if antigen is added to the antiserum prior to the addition of complement and erythrocytes modified by the corresponding antigen. The latter procedure, referred to as hemolysis inhibition test, allows the deter-

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mination of lipopolysaccharide in amounts as small as 0.006 μg. (7). In view of the important biologic acitivities of these endotoxins it was deemed desirable to determine the adsorptive capacity of activated attapulgite upon these lipopolysaccharides. The results of this investigation are embodied in the present report.

MATERIALS AND METHODS

Activated attapulgite was obtained from Wyeth Laboratories and dissolved just prior to use in phosphate hemagglutination buffer (pH 7.2–7.3) (Difco Laboratories).

As antigens, supernates of boiled agar grown cultures of *E. coli* 111:B4 and *Shigella* (*S.*) *sonnei* as well as purified lipopolysaccharides of *E. coli* 111:B4, 0127:B8 and *S. sonnei* were used. The two former lipopolysaccharides were obtained from Difco Laboratories and the latter through the kindness of Professor O. Westphal, Freiburg, Germany. All antigens were dissolved in phosphate buffer.

Mixtures of antigens and activated attapulgite and buffer, respectively, were prepared and incubated in a water bath at 37°, for one hour. The supernates of the mixtures (refrigerated centrifuge) were then used for modification of sheep red blood cells for the hemolysis tests and as antigens in the hemolysis inhibition tests. These methods have been described in detail elsewhere (4, 5, 8).

For the toxicity studies *E. coli* 127:B8 lipopolysaccharide was employed. The endotoxin was mixed with activated attapulgite and buffer, respectively. The mixtures were incubated in a water bath at 37° for one hour and centrifuged. White mice, weighing approximately 20 Gm., were injected intraperitoneally with 1 ml. each of the supernates. The animals were observed for a period of six days and fatal reactions recorded daily.

RESULTS

In the first series of experiments the effect of activated attapulgite on *E. coli* 0111:B4 lipopolysaccharide was determined, using the *E. coli* hemolysis test as indicator system. To this end, 5 μg./ml. of lipopolysaccharide was mixed with activated attapulgite in concentrations ranging from 17 to 5000 μg./ml. and buffer for control purposes. The mixtures were incubated, centrifuged, and supernates tested for remaining lipopolysaccharide by treatment of sheep red cells. The latter were washed and then added to *E. coli* 0111:B4 antiserum in serial dilutions and guinea pig complement. The resulting hemolysis was read and the results are recorded in Table I.

It can be seen from this table that activated attapulgite in concentrations of 500 μg./ml. and higher adsorbed enough lipopolysaccharide to prevent subsequent hemolysis completely and that 170 μg./ml. of this adsorbent reduced the amount of lipopolysaccharide sufficiently to produce partial inhibition of hemolysis. Similar results were obtained in experiments with *S. sonnei* lipopolysaccharide as well as with crude antigens.

The conclusion that activated attapulgite adsorbs endotoxins (lipopolysaccharides) is substantiated further by the results of experiments utilizing hemolysis inhibition as the indicator system. In these experiments various amounts of *E. coli* 0111:B4 lipopolysaccharide (1, 5, 25 μg./ml.) were mixed with activated attapulgite in concentrations of 100 to 10,000 μg./ml. and buffer for control purposes. The mixtures were incubated and centrifuged. The supernatant fluids were then used in hemolysis inhibition tests. Serial dilutions were mixed with homologous *E. coli* 0111:B4 antiserum; these mixtures were incubated at 37° for thirty minutes; sheep red blood cells modified by the identical lipopolysaccharide and guinea pig complement were then added. The results are recorded in Table II.

TABLE I.—EFFECT OF ACTIVATED ATTAPULGITE ON *Escherichia coli* LIPOPOLYSACCHARIDE SEROGROUP 0111

E. coli Antiserum	*E. coli* Lipopolysaccharide, 5 μg./ml., + Activated Attapulgite, μg./ml.						
	5,000	1,700	500	170	50	17	0
	Hemolysis						
1:100	–	–	–	2	3	3	4
1:500	–	–	–	–	2	3	3
1:2,500	–	–	–	–	–	–	–
0	–	–	–	–	–	–	–

– = No hemolysis; 1 to 4 = various degrees of hemolysis.

TABLE II.—EFFECT OF ACTIVATED ATTAPULGITE ON *Escherichia coli* SEROGROUP 0111 LIPOPOLYSACCHARIDE

E. coli Lipopolysaccharide, μg./0.1 ml.	0111 Lipopolysaccharide, μg./ml.											
	25				5				1			
	Plus Activated Attapulgite, μg./ml.											
	10,000	1,000	100	0	10,000	1,000	100	0	10,000	1,000	100	0
	Hemolysis Inhibition											
0.1	–	–	–	–	4	–	–	–	4	–	–	–
0.05	2	–	–	–	4	–	–	–	4	2	–	–
0.025	3	–	–	–	4	–	–	–	4	3	–	–
0.0125	4	–	–	–	4	±	–	–	4	4	–	–
0.006	4	3	–	–	4	3	2	1	4	4	2	±
0.003	4	4	–	–	4	4	3	3	4	4	3	3
0.0015	4	4	2	3	4	4	3	4	4	4	4	4
0	4	4	4	4	4	4	4	4	4	4	4	4

– = No hemolysis; 1 to 4 = various degrees of hemolysis.

Table II shows that activated attapulgite either reduces or abolishes the inhibitory effects of lipopolysaccharide. The higher the concentration of attapulgite and the lower the amounts of lipopolysaccharide used, the greater is the resulting effect. Essentially identical results were obtained in experiments with *E. coli* 0127:B8 and *S. sonnei* lipopolysaccharides as well as with crude antigens.

Experiments were undertaken to determine the effect of activated attapulgite on the toxicity in mice of endotoxin. For these experiments *E. coli* 0127:B8 lipopolysaccharide (1,000 μg./ml.) was mixed with activated attapulgite (5,000 μg./ml.) and buffer, respectively. The mixtures were incubated at 37° for one hour and centrifuged. The supernates were used for toxicity determinations by intraperitoneal injection of mice (1 ml. per animal). The results are summarized in Table III.

TABLE III.—EFFECT OF ACTIVATED ATTAPULGITE ON TOXICITY OF *Escherichia coli* 0127 ENDOTOXIN (LIPOPOLYSACCHARIDE)

Experiment	Groups of Mice Injected with Mixtures of Lipopolysaccharide, 1,000 μg./ml. and Attapulgite (Activated) 5,000 μg./ml. Ratio of Deaths/Number of Mice Injected After 2 days	6 days	Buffer 2 days	6 days
1	0/12	0/12	3/12	7/12
2	0/11	1/11	1/11	5/11
3	0/6	0/6	2/6	5/6
4	0/12	0/12	9/12	11/2
Total	0/41	0/41	15/41	28/41

Perusal of Table III shows that none of 41 mice injected with attapulgite treated lipopolysaccharide succumbed, whereas 15 out of 41 control mice died within two days and 28 within six days. The results clearly indicate that activated attapulgite adsorbs *E. coli* endotoxin as manifested by elimination of toxicity in mice.

DISCUSSION

The present investigation has revealed that activated attapulgite adsorbs endotoxins (lipopolysaccharides) from enteric bacteria, such as enteropathogenic *E. coli* and *S. sonnei*. It has been established, therefore, that this material adsorbs both exotoxins and endotoxins, the former represented by diphtheria toxin, a protein, and the latter by a lipopolysaccharide complex. Barr (9) reported that activated attapulgite is not a good adsorbent for enteric bacteria themselves, such as *Salmonella enteritidis* and *Shigella paradysenteriae*.

The effects of activated attapulgite on bacterial endotoxins is of interest from several points of view. In the first place, it is conceivable that such endotoxins play a role in localized intestinal infections. The fact that patients with bacillary dysentery and diarrheal disease due to enteropathogenic *E. coli* develop antibodies against the polysaccharide component of the endotoxin suggests that endotoxin in these illnesses reaches antibody forming cells beyond the lumen of the intestine. In this connection it should be mentioned that ingestion of "harmless" *E. coli* by human volunteers did not initiate antibody formation against the strain; in contrast, ingestion of enteropathogenic *E. coli* is followed by the appearance of specific antibodies (10). These observations suggest that, because of the very enteropathogenicity of the latter strains, absorption of endotoxin takes place. In the absence of intestinal infection, administration of Cr^{51}-labeled endotoxin did not result in detectable blood or tissue levels (11). It would be interesting to determine the effect of enteric infection on the absorption of labeled endotoxin. At the present time it is not known whether the endotoxin engendering antibody formation originates from the intestinal contents or from bacteria within or beyond the intestinal wall. If the former were the case, it is conceivable that activated attapulgite may possess therapeutic value. Only carefully controlled studies in patients will provide the answer to this moot question.

The capacity of activated attapulgite to adsorb endotoxin is of interest also because of various physiologic disturbances produced by these materials. For a summary the interested reader is referred to the review by Thomas (12). It suffices to mention here that these endotoxins (lipopolysaccharides) profoundly affect the nonspecific resistance to various infections, in part at least via the properdin system (3), contribute to irreversible shock (13), enhance nephrotoxicity of kidney antiserum (14), and exert antiallergic effects in animals (15).

SUMMARY

Activated attapulgite adsorbs endotoxins (lipopolysaccharides) from enteric bacteria, namely, enteropathogenic *Escherichia coli* and *Shigella sonnei*. This adsorption was demonstrated by three methods, the enterobacterial hemolysis test, the hemolysis inhibition test, and toxicity studies in mice.

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A Preliminary Phytochemical Investigation of *Digitalis Lutea* First Year Growth*

By JACK R. COLE† and OLE GISVOLD

The first year leaves of *Digitalis lutea* were extracted by two different techniques, one normal aqueous extraction and one which was enzyme inhibitory. Different results were obtained from each when examined by means of paper chromatography. A system of deacetylation and hydrolysis of the glycosides was developed involving the use of ion exchange resins. Several new solvent systems were developed for the paper chromatographic separation of sapogenins detected.

DIGITALIS LUTEA has been shown to be orally effective to a greater degree than some other members of the digitalis species (1). This fact and other research dealing with the yield of active cardiac glycosides (2–4) of some digitalis species stimulated an interest in the lutea species. Furthermore, since there has been a limited amount of phytochemical research in order to compare the nature of the constituents of the leaves of the consecutive years of growth of the Digitalis species, it was believed a study of this type might produce some interesting results.

The investigation of the second year growth of this plant has been reported (5).

EXPERIMENTAL

Harvesting, Storing, and Extraction Procedures.—The plants used in this study were grown during the summer of 1955 in the medicinal plant gardens at the University of Minnesota and collected in September, 1955. The storage procedure developed by Hopponen and Gisvold (2) was employed. Fresh leaves also were collected and used. Both lots yielded the same results. The general extraction method of Bay and Gisvold (6) was used to prepare a primary aqueous extract. In some cases emulsions are encountered when this aqueous primary extract is extracted with organic solvents. The use of 15% aqueous alcohol to prepare the primary extract substantially reduced the amount of emulsion that previously had been obtained. The primary extracts after an initial extraction with petroleum ether to remove fatty materials were then successively extracted with the following series of solvents: ether, ether:methylene dichloride 1:3, and methylene dicbloride.

The enzyme inhibitory extraction was adapted from that used by Stoll and Kreis (7). Table I, solvent system IV, indicates the chromatographic analysis of the material obtained from this extraction.

Paper Chromatographic Analysis.—The techniques employed by Gisvold and Schwartz (5) were followed for the most part. Slight modifications in the use of the solvent systems are indicated by the results in Table I.

The authentic samples of the glycosides were obtained from Dr. A. Stoll, Sandoz Co., Switzerland. Digoxigenin was obtained from Dr. Murphy, Analytical Research Laboratories, Burroughs Wellcome & Co., Tuckahoe, N. Y.

The comparison of the crude material obtained from the extraction of the leaves together with reference glycosides using solvent systems I, II, and III indicated the presence of acetyl digitoxin, digitoxin, and acetyl digoxin. In the case of solvent system IV, the presence of lanatoside A was distinctly indicated, and lanatoside B and C weakly present.

Deacetylation and Hydrolysis.—In order further to characterize the glycosides a system of deacetylation and hydrolysis followed by subsequent chromatography was carried out. The usual methods using alkali and acid for these studies are effective; however, the techniques may lead to undesirable changes. Furthermore, exact neutralization is very desirable and inorganic salts are introduced into the sample. In order to overcome some of the objections to the above techniques, ion exchange resins were investigated as a possible substitute for the acid or base.

Deacetylation.—For deacetylation studies, Amberlite resin IRA 400 (Rohm & Haas Co., Philadelphia) was chosen because it is a strongly basic exchange resin. It is a solid polystyrene quaternary amine that is insoluble in most solvents. All reactions should be employed at temperatures below 55–60°, since the resin loses catalytic activity rapidly and irreversibly at slightly higher temperatures. To convert the resin from the chloride form to the hydroxyl form, it is necessary to digest the resin with 1 *N* sodium hydroxide for approximately twelve hours. The resin is then washed with distilled water until the washings no longer give a basic test with Universal indicator papers. The resin mixture is then filtered and dried in the air.

Approximately 1 Gm. of the dry resin was placed in a test tube and covered with a solution of acetyl digitoxin in methanol (1 mg./cc.). This usually requires approximately 1.5 cc. of solution. The mixture was sealed and shaken frequently. At regular intervals of one hour, portions of this solution were drawn off and spotted on paper chromatograms. Samples of acetyl digitoxin and digitoxin also were spotted for comparison. The use of ascending chromatography was employed here

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TABLE I.—PAPER CHROMATOGRAPHY RESULTS OF THE CARDIAC GLYCOSIDES

Solvent System in Parts	I			II		III		IV
Methyl isobutyl ketone	5			20		5		..
Toluene	5			10		5		..
Formamide	*q. s.*			..		*q. s.*		..
Isopropyl ether	..			5		..		..
Ethylene glycol	..			10		..		..
Butanol	..			..		1		..
Ethylacetate	..			..		..		84
Benzene	..			..		..		16
Water	..			..		..		50
	A[a]	D[b]	H[c]	D	H	D	H	A
Development time (hours)	0.5	2.0	1.0	2.5	1.5	2.0	1.0	3.0
Glycoside	R_f Values							
Acetyl digitoxin	0.75	0.77	0.80	0.82	0.75	0.83	0.85	...
Acetyl digoxin	0.20	0.23	0.20	0.31	0.29	0.37	0.35	...
Digitoxin	0.46	0.44	0.46	0.59	0.56	0.56	0.61	...
Digoxin	0.10	0.10	0.09	0.13	0.15	0.24	0.25	...
Digoxigenin	...	...	...	...	...	0.34	0.30	...
Ditoxigenin	...	...	...	...	...	0.75	0.72	...
Lanatoside A	...	...	...	...	...	...	...	0.61
Lanatoside B	...	...	...	...	...	...	...	0.54
Lanatoside C	...	...	...	...	...	...	...	0.42

[a] Ascending. [b] Descending. [c] Horizontal.

as it serves as a rapid method of determining if any deacetylation had taken place. At the end of twelve hours, no reaction had taken place; however, after twenty-four hours some deacetylation had taken place. Substantial deacetylation was obtained after forty-eight hours.

A small column was substituted for a test tube and samples could be drawn off when desired. Deacetylation was obviously very slow and unreliable. When aqueous methanol (90%) was used in lieu of methanol and samples withdrawn every fifteen minutes, progressive deacetylation was obtained that was essentially complete after one hour.

The use of the resin was then applied to the plant extracts. The extract was treated in the same manner as described for the known compounds. A direct comparison was run on the same chromatogram. Acetyl digitoxin disappeared and a new spot, digitoxin, appeared signifying deacetylation had taken place. Solvent systems I, II, and III all were employed in these studies. Horizontal and descending methods were used.

Although the use of sodium hydroxide is more rapid, the use of the resin is certainly a more safe method and removes the danger of any impurities affecting later manipulation of the glycosides.

Ion Exchange Resin Hydrolysis.—For hydrolysis studies, Amberlite IR 120 (Rohm & Haas Co., Philadelphia) was chosen. This resin is a nuclear sulfonic acid type ion exchanger which has unusual stability at high temperatures. Immersion in boiling water appears to have no adverse reaction on the efficiency of the resin. The resin is completely insoluble in water, aliphatic and aromatic hydrocarbons, and most common solvents. It also is extremely stable in strongly acid and alkaline solutions. The resin is supplied in the sodium form and was converted to the hydrogen form by two successive rinses of 10% sulfuric acid. The resin is then washed with distilled water until the washings no longer turn Universal indicator paper acid.

The dry resin was placed in a column and covered with a methanolic solution of the glycoside of the usual concentration (1 mg./cc.). The solution was allowed to stand in contact with the resin for several days but apparently no hydrolysis had taken place. The use of water to speed the rate of hydrolysis was employed here as it had been successful in the deacetylation studies. The use of water did cause reaction to take place but this action was extremely slow. A full twenty-four hour period was required for hydrolysis. The resin-hydrolyzed compounds were run on paper chromatograms with both known aglycones and those produced by acid hydrolyses. They all exhibited the same R_f values.

The use of heat was employed in order to increase the rate of hydrolysis. The mixture of resin, digitoxin, and water was refluxed at 70° for fifteen minutes. It was then filtered and the resulting solution was spotted on paper with a known sample of digitoxigenin. Both compounds gave the same R_f value. This indicated that with the addition of water and heat the rate of the reaction can be increased quite substantially.

Digoxin was hydrolyzed in the same way but it required thirty minutes at 70° plus the addition of 15% water to cause complete hydrolysis.

Saponins.—The aqueous extracts of the leaves which had previously been extracted to remove the cardiac glycosides were next extracted with *n*-butanol to remove the saponins. Upon concentration of the butanol extract a solid separated that was labeled fraction I. Fraction II was obtained by complete evaporation of the butanol and as could be expected was quite impure. Fractions I and II contained saponins.

Fraction II was extracted with chloroform and a small amount of the material dissolved. A small amount of this chloroform solution was tested by means of the Raymond reaction and a positive cardiac glycoside test was obtained. The chloroform-soluble and chloroform-insoluble materials were recombined and chloroform removed by

TABLE II.—PAPER CHROMATOGRAPHIC RESULTS OF THE SAPOGENINS

Solvents Systems in Parts →	A[a]		B		C[b]	D	E	F	G	H	J
Petroleum ether (35–60°)	400		100		..	..	..	..	..	..	..
Ethanol	10		30		..	..	..	..	..	..	..
Water	90		70		..	..	..	..	..	..	..
Toluene	..		260		..	1	..	..	3	9	1
n-Butyl ether	..		..		*q. s.*	1	5	1	..	..	..
Propylene glycol (stationary phase)	..		..		*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	..
Methylisobutyl ketone	..		..		..	..	1	..	..	..	..
Petroleum ether (85–100°)	..		..		..	..	..	1	1	1	9
Samples	*Rf* Values										
Sapogenin I	0.10	0.56	0.54	1.0	..	..	..	..	..	..	..
Sapogenin II	0.10	0.55	0.56	1.0	..	..	..	..	..	..	..
Tigogenin	0.55		1.0		1.0	1.0	0.98	0.0	0.83	0.81	0.00
Sarsasapogenin	0.72		1.0		1.0	1.0	0.98	0.75	0.95	0.95	0.85
Gitogenin	0.09		0.55		0.4	0.61	0.43	..	..	..	..
Development time in hours	2		2		2.5	2.5	2.0	2.0	1.5	1.5	1.5

[a] Solvent system *A* and *B* (Heftmann, *et al.*): ascending.
[b] Solvent system *C* through *J*: horizontal.

evaporation. This material was then extracted with acetone as acetone dissolves glycosides readily, while saponins are insoluble. The residue remaining after the acetone extraction gave negative cardiac glycoside tests. The acetone extract was evaporated to dryness, purified, and chromatographed by means of solvent system IV. Lanatosides A, B, and C were determined to be present. This indicated incomplete extraction of the cardiac glycosides by the solvents used prior to extraction with butanol.

Paper Chromatography of the Sapogenins.—The saponins were hydrolyzed by means of hydrochloric acid in alcohol and water mixtures. The reaction mixtures were then extracted with chloroform, dried, and evaporated to dryness and used for chromatography.

The method of Heftmann and Hayden (8) was used for the preliminary chromatography of the sapogenins. However, since this system requires a twenty-four hour period of equilibration before development, other methods involving solvent equilibration of the paper was sought. The method of equilibrating the paper by means of saturation with organic solvents had been quite successful in the cardiac glycoside series. This method was applied to the chromatography of the sapogenins. Table II indicates the results of these studies. The standard sapogenins were obtained from Dr. G. Rosenkranz of Syntex Laboratories, Mexico City.

Solvent systems A and B indicated the presence of tigogenin and gitogenin. C, D, and E separated gitogenin from the other two sapogenins. F, G, H, and J separated sarsasapogenin and tigogenin. H was the most efficient system.

Some difficulty was encountered in these systems from tailing of the compounds. In solvent system C, tailing was appreciable. Solvent systems D, E, and H were practically free from this problem. F showed a small amount of tailing and G showed slightly more tailing; however, the separation was still obvious.

Identification of the Sugar Moities.—Mild hydrolysis of the saponins and subsequent paper chromatographic analysis (3) showed the presence of D-galactose, D-glucose, and D-xylose.

SUMMARY

1. Two extracts of the leaves of *Digitalis lutea* first year growth have been prepared by two different techniques. An enzyme inhibitory technique indicates the presence of the lanatosides A, B, and C. The second method consists of the preparation of aqueous and alcoholic-aqueous extracts and subsequent selective organic solvent extraction of these extracts. In this latter method, some enzymatic degradation has apparently taken place yielding the secondary glycosides.

2. Paper chromatographic analysis was the principal method of qualitative identification.

3. A system of deacetylation and hydrolysis of the glycosides was developed.

4. The saponins of *Digitalis lutea* were extracted with *n*-butanol, hydrolyzed with hydrochloric acid, and analyzed by paper chromatography. Tigogenin and Gitogenin were identified.

5. Some butanol-soluble glycosides of a polar nature not completely extracted previously were found.

6. Several new systems of paper chromatography have been developed for the rapid qualitative identification of the sapogenins.

7. Hydrolysis of the saponins and paper chromatographic investigation indicated the presence of D-galctose, D-glucose, and D-xylose.

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Some Physical Chemical Aspects of Suspension Formulation*

By TAKERU HIGUCHI

Difficulties inherent in treating problems associated with formulations of concentrated pharmaceutical suspensions are discussed from the standpoint of a physical chemist. Relationships which determine settling and creaming rates are treated in some detail, a new equation being proposed which may be more valid for heterodispersed, irregular particles than the classical Stoke's formula. Factors which influence crystal growth and caking are also discussed. Some reference is also made to diffusional processes of pharmaceutical importance.

A PHYSICAL CHEMIST assigned to problems concerning pharmaceutical suspensions is faced usually with a frustrating situation. His training, first of all, is largely in the basic areas and was designed to help him resolve problems dealing with only a few variables which can be expressed numerically. Unfortunately, pharmaceutical suspensions cannot be physically defined in terms of a limited number of these quantities.

In attempting to describe and define pharmaceutical suspensions, we do use terms expressible as numbers. Density of the internal phase, density of the external phase, phase volume ratio, viscosity of the external phase, etc., can all be described adequately and precisely with numbers. We also express variables such as particle size in terms of numbers but do so with considerable reluctance. The underlying statistics of the method employed, the degree of aggregation, the roughness of the particle surface, etc., strongly influence, in this case, our numerical answer. We make no attempt, on the other hand, to express variables such as size distribution with numbers. The problem furthermore becomes infinitely worse when we try to describe particle shapes precisely in terms of mathematics. This situation severely limits any comprehensive physical chemical approach to the general problem.

The situation, moreover, would not be greatly aided even if these difficult variables were of simpler nature. Unfortunately for physical chemists, we are not faced in these systems with thermodynamic but with kinetic problems. In a system at equilibrium, we are not particularly concerned with the mechanisms by which the end state had been achieved, the end results are always independent of the path taken. In suspension systems the primary concern is the rate of each physical process. How fast will it flow? What is the rate of settling (or creaming)? How long will it take for the suspended phase to crystallize or to grow? These are problems in kinetics and require reasonable understanding of the actual molecular process by which each phenomonon occurs, an understanding which we are largely lacking at the moment.

For these reasons, solutions to many problems involving pharmaceutical suspension have been obtained largely by the Edisonian trial and error approach. There are, nevertheless, real areas in which the basic, physical chemical thinking can be of major value. In the time available it is impossible to cover these in detail. For this reason only some of the major fields will be listed and a few points indicated to illustrate the nature of the problems involved.

RHEOLOGY

Rheological behavior of suspensions, especially those containing roughly more than 20% by volume of solids, is quite complex. *A priori* theoretical predictions of their flow properties are extremely difficult for reasons stated above. Results of some experimental studies in these areas will be presented by others in this series of papers.

SETTLING AND CREAMING

Difficulties associated with any attempt to relate particle properties and particle concentration to the settling or creaming rate of pharmaceutical suspensions serve as excellent examples of what can be done and what cannot be done with these problems. Let us see first how far simple theories together with simplifying assumptions will take us.

Following Stokes derivation, we can arrive at the familiar expression

$$v = \frac{ds}{dt} = \frac{2}{9} a^2 \frac{(\rho_1 - \rho_2)g}{\eta} \qquad \text{(Eq. 1)}$$

where the symbols have their usual meaning. In its formulation, we have, in attempting to apply it to real pharmaceutical suspension, assumed the following: (a) Particles are spherical (all of the same shape); (b) particles are all of the same size; (c) particles are sufficiently distant from each other so

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that the movement of one has no influence on its neighbors; (d) the rate is governed solely by hydrodynamic factors.

It is obvious that all of these assumptions are atrociously bad when considering real pharmaceutical systems. Except for the last, each seems worse than the others. The equation has no real value from a quantitative standpoint of predicting settling rates of the relatively concentrated suspensions such as those with which we are usually concerned. Any rigorous attempt to formulate the relationship in absence of these assumptions, on the other hand, seems to be doomed because of the mathematical complexity of the problem.

About ten years ago I considered this problem for relatively concentrated systems from what appeared to be a new approach. I did succeed in obtaining an equation which appears to be based on considerably fewer limitations. This derivation was based on an observation that despite the fact that pharmaceutical suspensions are usually heterodispersed, rather sharp lines of demarcation exist between the particle-free dispersion medium and the relatively concentrated suspension phase for concentrated suspensions which had stood undisturbed for some time. It appeared to me that for such a system the problem can be formulated as a movement of the external liquid phase moving through a bed of the solid internal phase. Fluid flow through packed beds has been treated mathematically by Kozeny and others. Such analyses have led to the Kozeny equation,

$$\mu = \frac{P}{L} \times 1/k\eta S_v^2 \times \frac{\epsilon^3}{(1-\epsilon)^2} \qquad \text{(Eq. 2)}$$

where μ is the linear flow velocity, P, the pressure drop across the bed, L, the bed thickness, k, the Kozeny constant approximately equal to 5, η, the viscosity of the permeating fluid, S_v, the specific surface area in sq. cm. per cm.^{3}, and ϵ, the porosity of the bed. There appears to be ample experimental evidence supporting the validity of this equation.

If we assume the relationship to be correct, we can readily derive the rate of creaming (or sedimentation) of suspensions. It can be shown that the pressure drop per unit depth of a uniform suspension acting to move the particles gravitationally is

$$P/L = (1-\epsilon)(\rho_1 - \rho_2)g \qquad \text{(Eq. 3)}$$

where $1 - \epsilon$ is the volume fraction of the internal phase. Substituting this into the Kozeny equation we get

$$\mu = \frac{(\rho_1 - \rho_2)g}{k\eta S_v^2} \frac{\epsilon^3}{1-\epsilon} \qquad \text{(Eq. 4)}$$

For suspensions of uniform spherical particles of radius, a, the equation simplifies to

$$u = \frac{(\rho_1 - \rho_2)ga^2}{9k\eta} \frac{\epsilon^3}{1-\epsilon} \qquad \text{(Eq. 5)}$$

These equations are strictly valid only during the initial phases in which ϵ is essentially constant near the moving boundary. It is also assumed in the development of these relationships that there is no change in the composition of the dispersed phase due to differential velocity of small and large particles. This can be justified partly on the basis that the composition of the dispersed phase near the moving boundary is maintained constant by the fact that small particles swept out of the suspension beyond the boundary would be replaced by particles coming in turn from the interior, and partly because of the caging effect of neighboring particles for these relatively concentrated systems—preventing relative motion of one particle with respect to another.

Comparison of Eqs. 1 and 5 shows a surprising degree of similarity. The factor $[(\rho_1 - \rho_2)ga^2]/9\eta$ is common to both. The Stokes law relation contains in addition only the factor *2* whereas the newly derived equation has the additional concentration factor, $\epsilon^3/[k(1-\epsilon)]$. The apparent similarity is somewhat misleading, however, since the physical processes basic to the two equations are quite different. It is obvious that the relationship based on the Kozeny equation only becomes valid for relatively concentrated systems where the suspended phase forms a sort of a bed. The simple Stokes equation on the other hand can only be used where each particle experiences essentially a free fall process.

In Fig. 1 the relative rate of settling for a suspension of uniform spheres of density = 2.0 and radius = 10μ is shown as a function of concentration (per cent phase volume) for Eqs. 1, 5, and for 1 corrected for phase ratio. The rate as predicted by the simple Stokes relationship is independent of the concentration of the internal phase. The equation when modified to correct for the increased flow rate within the bed as the result of decreased effective cross-sectional area of flow path yields a plot which shows a linear decrease in the settling rate with concentration. This plot obviously places an upper limit on the rate of sedimentation. Systems containing more than 10% by volume of the internal phase, according to Eq. 5, offer greater resistance to settling than that predicted by the Stokes equation. For these more concentrated suspensions the rate of settling decreases very rapidly with concentration, being roughly proportional to the fourth power of porosity for ϵ near 0.5.

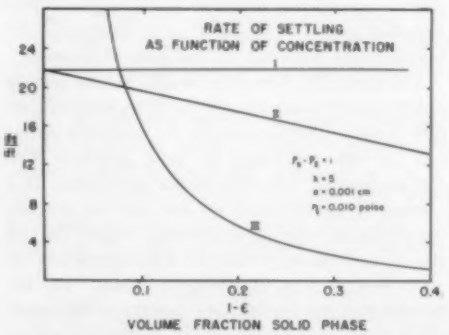

Fig. 1—Relative rates of settling predicted by several relationships are shown. Curve I was calculated directly from the classical Stokes equation. Curve II is the same as I except that it has been corrected for back flow of the external phase. Curve III represents that based on the Kozeny equation as explained in the text.

Although, in the figure, suspensions of uniform spheres were used as examples, the real value of the newly derived relationship is found in Eq. 4 which is for systems containing heterodispersed irregular particles. Since particle size does not occur explicitly in the formula and particle size distribution is not of concern, the equation can be applied directly to any suspensions for which Sv, is known. The latter can be determined readily by gas permeability or gas adsorption methods. Unfortunately no real test of the equation, derived has ever been carried out. This has been due to the feeling that the equations only treat the hydrodynamic aspects of the problem and totally neglect the contribution made to suspension stability arising from electrical and other particle-particle interactions. The treatment is thus valid for dispersion, for example, of hard solids in oils but probably is somewhat inadequate for aqueous suspensions of very small particles. This again illustrates the complexity of the problem of suspensions when viewed from theoretical standpoint.[1]

CRYSTAL GROWTH AND CAKING OF SEDIMENT

In practice a tendency of a suspension formula to settle or cream slowly does not prevent its adoption. Nearly all commercial products undergo this type of shelf deterioration to varying extents. Normally such suspensions are readily restored to their original states by moderate shaking. In many instances, however, redistribution of the sediment is not so easily effected because of the tendency of some crystals to knit together to form a hard cake. This behavior is often due to growth of the crystalline particles, especially at points of contact.

Increase in crystal size on storage is undesirable for other reasons. Larger particles are difficult to distribute uniformly. They tend to produce gritty texture unsuitable for topical, especially ophthalmic, use. These changes, moreover, result in marked alteration in rheological properties of the products.

The basic phenomenon of crystal growth has received very little attention from serious workers up to the present time, primarily because of the experimental difficulty associated with any attempt to measure the primary process. Although it is always due to growth of the more stable crystal forms at the expense of less stable components, the exact extent of this growth is extremely difficult to measure on real systems. Direct measurement of extent of caking of beds formed by settled suspension particles, moreover, presents even a greater problem. Since the causative process is fairly well established, however, it is possible to postulate from purely physical chemical and mechanistic standpoints some of the factors which may influence the overall process.

Cause.—Growth of a crystalline phase composed of material A is only possible if the thermodynamic activity of A in the solution phase is greater than that of the material in the crystals. The concentration of A, in other words, must be greater in the solution immediately in contact with the crystal than the solubility of the particular crystal. Such a supersaturated state can be reached by either dissolution of a thermodynamically more energetic form of A into the solution, or cooling of an already saturated solution.

It is evident, therefore, for suspension systems held at a constant temperature to undergo crystal growth that at least part of the suspended phase must be metastable. Since this part of the system would always possess greater solubility, the resulting saturated solutions would be supersaturated with respect to the stable configuration.

Such a metastable condition may arise as a result of a number of different situations. Thus amorphous or glassy preparations always exhibit significantly greater solubility than the corresponding crystalline forms. Most high molecular weight complex organic compounds exhibit polymorphic tendencies, the highest melting form being usually the stable modification. Even stable crystals can be rendered metastable by mechanical treatments which tend to increase the free energy content of these systems. Crushing and grinding operations usually yield particles whose different surfaces exhibit significantly higher or lower solubility. This behavior can be correlated directly with differences in the free surface energy of these powders introduced during the attrition process. Any crystalline flaw produced or an increase in the cumulative length of crystalline edges exposed will produce higher solubility.

As is well known, even perfect crystals differing in size will have different solubilities. Thus, as an example, for the following system: *a powder composed of cubic crystals uniformly 0.1μ along one edge having a density of 2.0, an average interfacial tension of 50 ergs/cm.² and made of substance having a molecular weight of 200*, we can calculate its relative solubility over that of coarse crystals from purely thermodynamic standpoint.

We can write $RT \ln(S/S_0) = \Delta F_s$, where S = solubility in the powder, S_0 = solubility of the gross crystals, and ΔF_s = difference in the molar free energy of those parts of the two systems which are involved in the dissolution equilibria.

This difference can be readily estimated by considering the relative effect on free energy of dissolution of infinitesimal amounts of the solid phase. Since the only energy difference between the large and small crystalline forms is in the surface energy we can derive the relation as follows: Let $A = 6L^2$ and $V = L^3$ where A, V, and L are the area, volume, and lineal dimension of the crystal under consideration. Then $dA = 12LdL$ and $dV = 3L^2dL$. But the corresponding free energy change, $dF = \gamma dA = 12L\gamma dL$ and $dF/dV = 4\gamma/L$. The rate of free energy change per mole would then correspond to the rate per unit volume as derived above multiplied by the molar volume of the solute;

$$\frac{dF}{dn} = \frac{M}{P}\frac{dF}{dV} = \frac{4\gamma m}{LP}$$

Introducing the free energy term into the solubility equation we find

$$RT \ln \frac{S}{S_0} = \gamma \left(\frac{1}{L} - \frac{1}{L_0}\right) \frac{4M}{P} \quad \text{(Eq. 6)}$$

[1] After completion of this manuscript, the author's attention has been called to a similar treatment by P. G. W. Hawksley, "Some Aspects of Fluid Flow," Arnold, London, 1951. Mr. Hawksley also has utilized the porous bed approach and has obtained good agreement with experimental data.

Substituting in the numerical quantities and neglecting $1/L_0$ since it would be expected to be much smaller than $1/L$, we find $\ln (S/S_0) = 0.08$ at room temperature, or roughly 8% higher solubility for the powder. For crystals tenfold larger, the increase is only 1%; for those only one tenth as large, i. e., 0.01μ on edge, solubility is 2.2 times that of the coarse crystals. It is evident that the effect becomes significant for only very small particles.

Some Possible Approaches to Prevention.—Crystal growth and caking of sediments when due to growth can be largely prevented or reduced in many cases by proper attention to certain fundamentals. The approach can be made either thermodynamically or kinetically.

Since the cause of crystal growth is due largely to the presence of particles at energetic states higher than that of the stable form any and all steps taken which would tend to minimize this energy difference should result in greater stability of the product. Thus selection of the stable crystalline modification in formulating, preparation of powders for suspensions without mechanical comminution, and use of particles of relatively narrow particle size ranges are indicated. Another possibly useful method is based on reducing the free surface energy of the interface between the particle surface and the solvent phase. It is evident from Eq. 6 that if γ were made very small, the effect of particle size could be rendered negligible. It is conceivable that addition of selected surface active agents can readily reduce interfacial tension of most organic drugs against water, for example, to less than 10 dyne/cm.

Even though a suspension system is essentially homogeneous from energetic standpoint, some periodic dissolution and recrystallization may be expected as a result of temperature fluctuations during storage. For these systems and for cases where inclusion of a metastable form cannot be obviated, satisfactory increase in stability can often be achieved by retarding the overall growth process. Rate of both growth and nucleations of the stable form can be reduced, for example, by increasing the viscosity of the suspending medium. Since the kinetics of both processes is largely determined by the diffusion rate, the overall rate would be expected to be approximately an inverse function of viscosity. Thus a tenfold increase in the viscosity of the suspending fluid should result in a corresponding increase in shelf life.

Another solution to the problem makes use again of surface active agents. If a layer of foreign material is adsorbed on the surface of the stable crystalline surface the rate of deposition of solute molecules is greatly decreased. Under certain conditions growth can be totally inhibited even when in contact with a supersaturated solution. At the same time the rate of solution of the more soluble particles would be correspondingly diminished by the same film. Certain proteins which may undergo some denaturation at the interface seem to be particularly efficacious for this purpose.

DIFFUSIONAL BEHAVIOR OF SUSPENDED DRUGS

This is an extremely interesting area of study for physical chemists as many model situations can be set up and solved mathematically. A good deal of attention has already been directed toward the more basic aspects of the problem and has permitted intelligent application of theories to practical problems.

Since this short survey is hardly adequate to treat the entire field, I have listed below a few types of diffusional problems pertaining to suspensions for which mathematical solutions are presently available.

1. Diffusion of drugs from a finite thickness of a thick suspension layer into a constantly depleted layer, the drug being dissolved in the external phase.
2. Same as 1, except that the drug is now the suspended phase.
3. Diffusion of drug into and through a barrier of finite thickness containing a suspended phase having an affinity for the drug different than the external phase.

Many others of similar type can and have been readily solved.

GENERAL REMARKS

From the preceding examples and discussions it is apparent that physical chemists must choose their ground of operation very carefully if they are to contribute significantly to such applied problems as suspension formulation. As is evident, there are large areas related to the pharmaceutical systems of interest in which we can make good use of our educational background. We must realize, on the one hand, that there are certain aspects concerning which the basic theories are so inadequate that experimentation alone can give us the answer. On the other, we must make the best of unsatisfactory situations. Unlike classical physical chemists we must stand ready to work with messy, complex systems in which many of the variables are not controlled.

The Effect of Vehicle and Particle Size on the Absorption, by the Intramuscular Route, of Procaine Penicillin G Suspensions*

By F. H. BUCKWALTER and H. L. DICKISON

Serum concentrations in rabbits following intramuscular injection of numerous suspensions of procaine penicillin G in various vehicles have been presented. The effects of the nature of the vehicle and the particle size of the procaine penicillin G have been studied. The results indicate that the most practical formulation for delayed absorption is 300,000 units of small particle (less than 5μ) procaine penicillin G per cc. of peanut oil or sesame oil gelled with 2% aluminum monostearate. Extensive data in human subjects are submitted to corroborate the animal results.

A NEW absorption delaying vehicle for penicillin has been reported by Buckwalter and Dickison (1). A formulation using this vehicle and procaine penicillin G of small particle size (particles less than 5 μ) has been used for several years as standard treatment for many of the treponemal diseases.

Very little is known about the mechanism of absorption of drugs after any route of administration. Actual determination of the concentration of a drug in the blood stream was virtually unpracticed until the advent of the sulfonamides. Marshall (2) introduced the method for the determination of sulfonamides in blood. With the introduction of penicillin and other antibiotics, the determination of serum concentrations came into widespread use.

These determinations indicated that following intramuscular injections of aqueous solutions of sodium or potassium penicillin G rapid absorption occurred with the production of high serum levels of penicillin. These high serum concentrations decreased rapidly in a few hours due to the rapid excretion of penicillin in the urine after its removal from the site of injection. It became desirable to prevent this rapid absorption with its attendant high serum concentration and to spread the absorption over longer periods of time thus maintaining therapeutic serum concentrations for days instead of hours.

The absorption of penicillin following intramuscular injection could be influenced by the following:

(a) The nature of the penicillin salt used.—In general the more soluble the salt the more quickly it will be absorbed and vice versa. The relatively insoluble salts, procaine penicillin G and benzathine penicillin G give much slower absorption than the soluble potassium and sodium penicillins.

(b) The nature of the vehicle used.—In general, oil vehicles slow up absorption and aqueous vehicles speed absorption. The field of vehicles for intramuscular use is still virtually unexplored.

(c) Crystal form of the penicillin salt used.—This is rather virgin territory but there does seem to be an effect, which is still undetermined, of crystal form on absorption.

(d) Particle size and particle size distribution.—This is very probably a most important aspect of absorption. Generally large particles are more slowly absorbed than small particles but there are noteworthy exceptions to this.

(e) Coating of the particles of the drug for delaying absorption.

(f) Viscosity of the product.—Viscosity *per se* does not appear to influence absorption since some rather viscous products are absorbed faster than less viscous products.

EXPERIMENTAL

During the work on repository penicillin we compiled a tremendous amount of data on both rabbits and man. This report covers only some of the more salient points and it has been necessary to leave out entirely a good share of the work. As just one example, we can say that zirconium and germanium stearates behave the same as aluminum stearate when gelled with vegetable oils and delay absorption whereas calcium, zinc, and magnesium stearates appear to have very little or no effect on the rate of absorption.

The work presented represents the results obtained following intramuscular injections of various formulations in rabbits and man. In all of the rabbit studies, intramuscular injection of 50,000 units/Kg. were made into the thigh muscles, since we have found that this dosage in rabbits correlates rather closely with the results obtained following the intramuscular injection of 300,000 units into man. In the rabbit studies, a minimum of 5 rabbits was used for each formulation so that all data given represent the average of the results obtained with at least 5 rabbits. Each human subject received a single intramuscular injection of 300,000 units deep

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into the gluteal muscle. In both the animal and human work, we considered 0.03 unit of penicillin per cc. of plasma as the theoretical minimum effective concentration. We have found in both rabbits and humans that the percentage of rabbits or humans showing a blood level of 0.03 unit or higher at any stated hour is a most important criterion for a repository product. In many formulations there has been found a good repository effect in a portion of the animals or humans but the percentage was too low for a practical repository product. Many formulations tested showed an average blood level at seventy-two or ninety-six hours of greater than 0.03 unit but the percentage of patients who showed a blood level of 0.03 unit or higher was less than 50%. In this work there was used a requirement or criterion that a good repository product must not only show an average blood level of 0.03 unit or higher but 80% or more of the patients must also show 0.03 unit or higher at whatever hour that might be claimed for the product.

RESULTS

Table I represents a comparison of rabbit blood levels using micronized procaine penicillin G in 11 different vehicles. These vehicles included sesame oil alone, sesame oil gelled with 1 to 5% aluminum monostearate and sesame oil with 1 to 5% beeswax. The procaine penicillin G used had 95% of the particles below 5 μ and essentially 100% of the particles were less than 10 μ. These data indicate that aluminum monostearate has a very considerable effect in delaying absorption whereas beeswax has only a slight effect.

Table II represents a comparison of rabbit blood levels using 100 mesh procaine penicillin G in the same 11 vehicles described above. The 100 mesh procaine penicillin G represented material which went through an 80-mesh screen but was retained on a 100-mesh screen so that the material had a particle size distribution of approximately 150–175 μ. Microscopic examination corroborated this approximate particle size range. These data indicate that these relatively large particles tend to delay to some extent absorption in the oil alone and in the oil-beeswax vehicles but appear to accelerate absorption in the aluminum monostearate vehicles.

Table III represents a comparison of rabbit blood levels using 250 mesh procaine penicillin G in the same 11 vehicles described above. The 250 mesh procaine penicillin G represented material which went through a 250-mesh screen but was retained on a 325-mesh screen so that the material had a particle size distribution of approximately 45–60 μ. Microscopic examination corroborated this approximate particle size range. These data indicate that these relatively small particles are absorbed rather rapidly in the oil alone and in the oil-beeswax vehicles but are absorbed rather slowly from the aluminum monostearate vehicles.

The entire series represented in Tables I, II, and III were repeated using peanut oil in place of sesame oil. The results obtained were quite similar to those obtained with sesame oil. There is apparently no significant difference between the two oils in so far as absorption is concerned.

Table IV represents a condensation of some of the results in Tables I, II, and III. It shows the effect of particle size of the procaine penicillin G in sesame oil, in sesame oil gelled with 2% aluminum monostearate, and in sesame oil containing 5% beeswax. These data indicate that large particles of procaine

TABLE I.—PENICILLIN SERUM CONCENTRATIONS IN RABBITS.
MICRONIZED PROCAINE PENICILLIN G (300,000 UNITS/CC.) IN SESAME OIL VEHICLES

Vehicle	Normal	Average Blood Levels, Hours											
		1	4	24	48	72	96	120	144	168	192	216	240
Sesame Oil	NR	5.53	2.27	0.03	NR	..	..	..	..	..	..	..	..
+ 1% ALMST[a]	NR	0.84	0.85	0.51	0.45	0.35	0.07	0.02	NR	..	..	..	..
+ 2% ALMST	NR	1.03	0.71	0.33	0.26	0.10	0.09	0.05	0.04	0.06	0.02	NR	..
+ 3% ALMST	NR	3.39	1.52	1.03	0.69	0.20	0.13	..	0.02	0.08	0.02	NR	..
+ 4% ALMST	NR	1.53	2.46	0.87	0.28	0.11	0.04	0.08	0.08	0.06	NR	..	..
+ 5% ALMST	NR	1.26	1.01	0.56	0.97	0.20	0.03	0.05	0.02	0.015	0.03	0.02	NR
+ 1% Beeswax	NR	1.93	1.78	0.33	0.10	NR	..	..	..	..	..	..	..
+ 2% Beeswax	NR	2.18	1.78	0.38	0.07	0.07	0.02	NR	..	..	..	..	..
+ 3% Beeswax	NR	2.36	1.58	0.11	0.06	NR	..	..	..	..	..	..	..
+ 4% Beeswax	NR	4.35	1.76	0.35	0.01	NR	..	..	..	..	..	..	..
+ 5% Beeswax	NR	4.14	2.28	0.28	0.07	NR	..	..	..	..	..	..	..

[a] ALMST = Aluminum monostearate. NR = No zones of inhibition.

TABLE II. PENICILLIN SERUM CONCENTRATIONS IN RABBITS.
100 MESH (LARGE PARTICLE) PROCAINE PENICILLIN G (300,000 UNITS/CC.) IN SESAME OIL VEHICLES

Vehicle	Normal	Average Blood Levels, Hours											
		1	4	24	48	72	96	120	144	168	192	216	240
Sesame Oil	NR	3.50	1.45	0.16	0.05	NR	..	..	..	..	..	..	..
+ 1% ALMST	NR	2.05	1.66	0.25	0.09	0.04	0.02	0.008	NR	..	..	..	..
+ 2% ALMST	NR	2.58	1.83	0.21	0.04	0.01	NR	0.04	NR	..	..	..	..
+ 3% ALMST	NR	2.24	1.27	0.24	0.11	0.16	0.11	0.01	0.01	0.02	NR	..	0.02
+ 4% ALMST	NR	1.90	1.01	..	..	0.14	0.16	0.05	0.03	0.03	..	..	0.11
+ 5% ALMST	NR	1.88	1.05	..	..	0.15	0.08	0.05	0.03	0.02	..	..	0.01
+ 1% Beeswax	NR	4.13	2.58	0.015	NR	NR	..	..	..	..	..	..	..
+ 2% Beeswax	NR	5.58	4.13	0.15	0.04	0.008	NR	..	..	..	..	..	..
+ 3% Beeswax	NR	2.61	1.92	0.17	0.04	0.008	NR	..	..	..	..	..	..
+ 4% Beeswax	NR	3.71	2.68	0.10	0.03	NR	0.01	NR	..	..	..	..	..
+ 5% Beeswax	NR	3.66	2.84	0.11	0.05	0.008	NR	NR	..	..	..	..	..

TABLE III.—PENICILLIN SERUM CONCENTRATIONS IN RABBITS.
250 MESH PROCAINE PENICILLIN G (300,000 UNITS/cc.) IN SESAME OIL VEHICLES

Vehicle	Normal	Average Blood Levels, Hours											
		1	4	24	48	72	96	120	144	168	192	216	240
Sesame Oil	NR	2.78	1.88	0.07	0.01	NR	..	..	..	..	..	..	..
+ 1% ALMST	NR	2.68	1.80	0.80	0.07	0.005	0.015	0.005	NR	NR	..	..	..
+ 2% ALMST	NR	3.03	2.20	0.08	0.03	0.04	0.06	0.045	NR	..	..	..	..
+ 3% ALMST	NR	2.51	1.71	0.23	0.10	0.17	0.06	0	..	0.04	0.03	0.03	0.03
+ 4% ALMST	NR	2.99	1.31	0.32	0.09	0.12	0.05	0	..	0.008	0.005	NR	NR
+ 5% ALMST	NR	1.56	0.85	0.31	0.16	0.18	0.05	0.02	0.02	0.03	0.05	0.015	0.04
+ 1% Beeswax	NR	4.13	2.65	0.22	0.04	NR	NR	0	..	..	..	..	..
+ 2% Beeswax	NR	2.80	2.08	NR	..	..	0	0	..	..	..	..	..
+ 3% Beeswax	NR	4.25	1.93	0.18	0.008	NR	..	0	..	..	..	..	..
+ 4% Beeswax	NR	2.69	1.55	0.26	0.09	0.02	0.01	NR	..	..	..	..	..
+ 5% Beeswax	NR	3.04	1.23	0.16	0.07	0.04	NR	..	NR	..	..	..	..

TABLE IV.—PENICILLIN SERUM CONCENTRATION IN RABBITS.
COMPARISON OF PARTICLE SIZE VS. VEHICLE—PROCAINE PENICILLIN G (300,000 UNITS/cc.) SUSPENSIONS

Vehicle	Particle Size of Proc. Pen. G	Normal	Average Blood Levels, Hours										
			1	4	24	48	72	96	120	144	168	192	216
Sesame Oil	Micronized	NR	5.53	2.27	0.03	NR	..	..	..	..	..	..	..
Sesame Oil	100 Mesh	NR	3.50	1.45	0.16	0.05	NR	..	..	..	..	..	..
Sesame Oil	250 Mesh	NR	2.78	1.88	0.07	0.01	NR	..	..	..	..	..	..
+ 2% ALMST	Micronized	NR	1.03	0.71	0.33	0.26	0.19	0.09	0.05	0.04	0.06	0.02	NR
+ 2% ALMST	100 Mesh	NR	2.58	1.83	0.21	0.04	0.01	NR	0.04	NR	..	..	..
+ 2% ALMST	250 Mesh	NR	3.03	2.20	0.08	0.03	0.04	0.06	0.045	NR	..	..	..
+ 5% Beeswax	Micronized	NR	4.14	2.28	0.28	0.07	NR	..	..	..	..	..	..
+ 5% Beeswax	100 Mesh	NR	3.66	2.84	0.11	0.05	0.008	NR	NR	..	..	..	..
+ 5% Beeswax	250 Mesh	NR	3.04	1.23	0.16	0.07	0.04	NR	..	..	..	..	..

penicillin G delay absorption in oil and in oil-beeswax systems but the exact reverse is found in the sesame oil gelled with aluminum monostearate systems where small particles delay absorption. The delayed absorption is much greater with small particles in the oil-aluminum stearate systems than can be obtained with large particles in the oil or oil plus beeswax systems.

Table V represents a comparison of rabbit blood levels using aqueous suspensions of procaine penicillin G with a range of particle size distribution of the procaine penicillin G. It is readily apparent from these data that large particles of procaine penicillin G delay absorption from aqueous systems whereas small particles accelerate absorption.

Table VI represents rabbit blood levels obtained with micronized (<5 μ) procaine penicillin G in peanut oil gelled with 2 to 5% aluminum monostearate at concentrations of 300,000 and 400,000 units/cc. There is some prolongation of absorption with the higher concentrations of penicillin and with amounts of aluminum monostearate greater than 2%. However, the viscous nature of these formulations makes them impractical for commercial use.

TABLE V.—PENICILLIN SERUM CONCENTRATION IN RABBITS.
AQUEOUS SUSPENSIONS OF PROCAINE PENICILLIN G (300,000 UNITS/cc.)

Vehicle	Mesh Size	Particle Size in Microns	Average Blood Levels, Hours					
			1	4	24	28	48	72
Water	60–100	150–250	1.37	1.29	0.82	0.86	0.31	0.12
Water	100–140	105–150	1.24	1.50	0.76	0.28	0.16	0.01
Water	140–250	58–105	1.54	1.44	0.47	0.25	0.12	0.04
Water	250–400	35–38	1.64	1.51	0.62	0.33	0.15	NR
Water	400	<35	2.40	2.36	0.33	0.16	0.07	NR
Water	Micronized	1–2	2.14	2.22	0.06	0.02	NR	NR

TABLE VI.—PENICILLIN SERUM CONCENTRATION IN RABBITS.
MICRONIZED PROCAINE PENICILLIN G USED

Vehicle Peanut Oil	Concentration of Product	Average Blood Levels, Hours												
		1	24	48	72	96	144	168	192	216	240	264	288	312
+ 2% ALMST	300,000/cc.	0.57	0.60	0.34	0.24	0.10	0.02	0.014	0.012	0.004	..	..	..	..
+ 3% ALMST	300,000/cc.	1.11	0.68	0.26	0.13	0.08	0.03	0.015	0.008	..	..	NR	..	..
+ 4% ALMST	300,000/cc.	1.15	0.35	0.14	0.19	0.14	0.07	0.06	0.08	0.03	..	0.01	..	..
+ 5% ALMST	300,000/cc.	1.11	0.33	0.19	0.25	0.23	0.09	0.06	0.04	0.03	0.015	0.005	..	..
+ 2% ALMST	400,000/cc.	2.22	0.76	0.36	0.33	0.18	0.08	0.03	0.02	..	..	0.01	0.008	NR
+ 3% ALMST	400,000/cc.	1.03	0.52	0.32	0.27	0.22	0.13	0.07	0.05	0.025	0.025	0.02	0.01	0.03
+ 4% ALMST	400,000/cc.	1.24	0.35	0.13	0.13	0.23	0.15	0.11	0.09	0.04	0.05	0.02	..	0.008
+ 5% ALMST	400,000/cc.	1.02	0.41	0.22	0.26	0.28	0.22	0.138	0.07	0.07	..	0.04	0.005	..

Table VII represents rabbit blood levels obtained with 250 mesh procaine penicillin G (45–60 μ) in peanut oil gelled with 2 to 5% aluminum monostearate at concentrations of 300,000, 400,000, and 500,000 units/cc. These larger particles of procaine penicillin G show faster absorption than that obtained with the smaller, micronized particles of procaine penicillin G. There is some prolongation of absorption with the higher concentrations of penicillin and with amounts of aluminum monostearate greater than 2%. However the viscous nature of these formulations makes them impractical for commercial use.

Tables VIII and IX represent rabbit blood levels obtained with micronized (<5 μ) and 100 mesh (150–175 μ) procaine penicillin G in peanut oil and in peanut oil gelled with varying amounts (0.1 to 10%) of aluminum monostearate. These data indicate that aluminum monostearate materially delays absorption and that small particles are much more effective in delaying absorption than large particles. Amounts of aluminum monostearate greater than 2% further delay absorption but the formulations are too viscous for practical use.

Tables X, XI, and XII represent the rabbit blood levels with micronized (<5 μ) and large (75–150 μ) particles of procaine penicillin G from four different crystal types of procaine penicillin G in the same vehicle. Three vehicles are used, namely (a) peanut oil gelled with 2% aluminum monostearate, (b) peanut oil gelled with 2% aluminum distearate, and (c) peanut oil gelled with 2% aluminum tristearate. These data indicate that, without doubt, small particles are much better for delaying absorption from these vehicles. However, the type of procaine penicillin G used also has an effect on absorption. This effect is not clear and needs further investigation. There is also the possibility that the type of stearate used (mono-di-tri) may have some effect on absorption but if all other conditions are equal, it is believed this effect will be negligible.

Table XIII represents the blood levels obtained in human subjects with a suspension of procaine penicillin G in peanut oil. This formulation gives, at best, only twenty-four hour blood levels.

Table XIV represents the blood levels obtained in human subjects with a suspension of large parti-

TABLE VII.—PENICILLIN SERUM CONCENTRATION IN RABBITS. 250 MESH PROCAINE PENICILLIN G USED

Vehicle	Concentration of Product	Average Blood Levels, Hours 1	24	48	72	96	120	144	168	192	264	288	213	336
Peanut Oil														
+ 2% ALMST	300,000/cc.	6.62	0.41	0.11	0.03	0.004	..	..	..	..	..	..	..	..
+ 3% ALMST	300,000/cc.	4.50	0.22	..	..	0.05	0.01	NR	..	..	..	..	..	..
+ 4% ALMST	300,000/cc.	2.16	0.41	..	..	0.09	0.05	0.05	0.03	0.02	NR	..	..	..
+ 5% ALMST	300,000/cc.	2.18	0.29	..	..	0.14	0.07	0.06	0.04	0.02	NR	..	..	..
+ 2% ALMST	400,000/cc.	4.40	0.55	..	..	0.05	0.02	0.02	0.01	0.008	NR	..	..	..
+ 3% ALMST	400,000/cc.	3.50	0.30	..	..	0.15	0.10	0.04	0.02	NR	..	..	..	..
+ 4% ALMST	400,000/cc.	3.14	0.36	..	..	0.09	0.06	0.03	0.03	0.03	NR	..	..	..
+ 5% ALMST	400,000/cc.	2.18	0.32	..	..	0.11	0.08	0.08	0.05	0.03	NR	..	..	..
+ 2% ALMST	500,000/cc.	5.38	0.94	..	..	0.12	0.08	0.08	0.06	0.03	NR	..	..	..
+ 3% ALMST	500,000/cc.	2.58	0.37	..	..	0.08	0.05	0.03	0.03	0.02	0.02	0.01	0.01	0.006
+ 4% ALMST	500,000/cc.	4.40	0.83	..	..	0.07	0.03	0.02	0.008	NR	..	..	..	..
+ 5% ALMST	500,000/cc.	2.13	0.45	..	..	0.09	0.08	0.04	0.06	0.03	0.02	0.01	0.03	0.03

TABLE VIII.—PENICILLIN SERUM CONCENTRATIONS IN RABBITS. MICRONIZED PROCAINE PENICILLIN G (300,000 UNITS/cc.)

Vehicle	Average Blood Level, Hours 1	4	24	48	72	96	120	144	168	192	216	240	264
Peanut Oil	3.70	2.50	0.01	NR	..	..	..	..	..	..	..	..	..
+ 0.1% ALMST	3.60	1.70	0.25	0.01	NR	..	..	..	..	..	..	..	..
+ 1% ALMST	1.40	1.30	0.32	0.11	0.01	NR	..	..	..	..	..	..	..
+ 2% ALMST	1.45	0.63	0.25	0.25	0.30	0.04	0.05	NR	..	..	..	..	..
+ 3% ALMST	0.99	0.40	0.41	0.26	0.26	0.24	0.11	0.06	0.02	0.01	0.01	NH	..
+ 5% ALMST	0.74	0.44	0.38	0.40	0.26	0.24	0.11	0.05	0.01	0.01	NR	..	..
+ 10% ALMST	0.60	0.21	0.21	0.13	0.10	0.12	0.06	0.05	0.03	0.01	0.02	0.01	0.01

TABLE IX.—PENICILLIN SERUM CONCENTRATIONS IN RABBITS. 100 MESH PROCAINE PENICILLIN G (300,000 UNITS/cc.)

Vehicle	Average Blood Level, Hours 1	4	24	48	72	96	120	144	168	192	216	240	264
Peanut Oil	2.97	1.80	0.07	NR	..	..	..	..	..	..	..	..	..
+ 0.1% ALMST	3.00	2.07	0.24	0.06	NR	..	..	..	..	..	..	..	..
+ 1% ALMST	3.00	1.40	0.28	0.14	0.02	NR	..	..	..	..	..	..	..
+ 2% ALMST	2.98	1.92	0.31	0.39	0.03	NR	..	..	..	..	..	..	..
+ 3% ALMST	1.97	1.01	0.60	0.26	0.16	0.07	0.04	0.01	0.01	0.01	0.01	..	..
+ 5% ALMST	1.42	0.69	0.27	0.26	0.22	0.13	0.07	0.02	0.02	0.02	0.02	0.01	0.01
+ 10% ALMST	0.60	0.31	0.21	0.59	0.23	0.18	0.07	0.07	0.03	0.01	..	..	..

TABLE X.—PENICILLIN SERUM CONCENTRATIONS IN RABBITS.
SUSPENSIONS OF PROCAINE PENICILLIN G (300,000 UNITS/CC.) IN PEANUT OIL + 2% AL MONOST

Type of Proc. Pen. Crystal	Particle Size	Average Blood Levels, Hours							
		1	4	24	48	72	96	120	144
Small Type From Propanol	100–200 mesh	1.20	0.66	0.69	0.32	0.26	0.15	0.10	0.02
Small Type From Propanol	Micronized	0.76	0.46	0.45	0.37	0.15	0.13	0.07	0.04
Large Type From Propanol	100–200 mesh	2.54	1.80	0.28	0.13	0.10	0.06	0.03	0.02
Large Type From Propanol	Micronized	2.19	1.23	0.50	0.15	0.06	0.03	0.02	0.01
Acetone-Water	100–200 mesh	0.64	0.41	0.56	0.40	0.18	0.14	0.05	0.04
Acetone-Water	Micronized	0.85	0.49	0.36	0.23	0.14	0.07	0.01	..
Water Precipitated	100–200 mesh	2.94	1.09	0.38	0.25	0.09	0.06	0.05	0.02
Water Precipitated	Micronized	1.18	0.73	0.50	0.32	0.20	0.11	0.07	0.04

TABLE XI.—PENICILLIN SERUM CONCENTRATIONS IN RABBITS.
SUSPENSIONS OF PROCAINE PENICILLIN G (300,000 UNITS/CC.) IN PEANUT OIL + 2% AL DIST

Type of Proc. Pen. Crystal	Particle Size	Average Blood Levels, Hours							
		1	4	24	48	72	96	120	144
Small Type From Propanol	100–200 mesh	1.26	0.74	0.54	0.42	0.23	0.18	0.10	0.06
Small Type From Propanol	Micronized	0.66	0.34	0.31	0.30	0.19	0.13	0.05	0.04
Large Type From Propanol	100–200 mesh	2.24	1.80	0.40	0.24	0.14	0.09	0.03	0.02
Large Type From Propanol	Micronized	1.21	0.86	0.51	0.24	0.14	0.11	0.08	0.02
Acetone-Water	100–200 mesh	1.17	0.68	0.62	0.40	0.12	0.07	0.07	0.02
Acetone-Water	Micronized	0.59	0.38	0.45	0.47	0.15	0.03	NR	..
Water Precipitated	100–200 mesh	2.22	1.01	0.60	0.18	0.07	0.02	0.005	..
Water Precipitated	Micronized	0.76	0.45	0.44	0.27	0.19	0.12	0.11	0.05

TABLE XII.—PENICILLIN SERUM CONCENTRATIONS IN RABBITS.
SUSPENSIONS OF PROCAINE PENICILLIN G (300,000 UNITS/CC.) IN PEANUT OIL + 2% AL TRIST

Type of Proc. Pen. Crystal	Particle Size	Average Blood Levels, Hours							
		1	4	24	48	72	96	120	
Small Type From Propanol	100–200 mesh	1.65	0.85	0.54	0.27	0.20	0.11	0.07	0.06
Small Type From Propanol	Micronized	0.88	0.42	0.33	0.21	0.15	0.09	0.03	0.02
Large Type From Propanol	100–200 mesh	2.35	1.47	0.30	0.18	0.11	0.06	0.03	0.02
Large Type From Propanol	Micronized	1.29	0.90	0.34	0.29	0.15	0.15	0.10	0.04
Acetone-Water	100–200 mesh	1.63	0.72	0.54	0.40	0.24	0.09	0.02	0.003
Acetone-Water	Micronized	0.52	0.34	0.28	0.34	0.33	0.17	0.02	0.01
Water Precipitated	100–200 mesh	1.45	0.73	0.41	0.18	0.12	0.09	0.08	0.14
Water Precipitated	Micronized	0.70	0.64	0.29	0.22	0.17	0.13	0.09	0.17

TABLE XIII—PENICILLIN SERUM CONCENTRATION IN HUMANS.
PROCAINE PENICILLIN G IN PEANUT-OIL—PARENTERAL[a]

Time, Hr.	Number of Patients	Average Blood Level, Units/ml.	Patients With 0.03 or Higher, %
1	50	1.09	100
12	50	0.218	92
24	49	0.051	33
30	46	0.021	20
36	46	0.01	11
40	46	0.005	11
48	46	0.002	2

[a] Dose—1 cc. I. M.—300,000 units of procaine penicillin G per cc. of peanut oil.

TABLE XIV—PENICILLIN SERUM CONCENTRATION IN HUMANS.
PROCAINE PENICILLIN G (LARGE PARTICLES) IN PEANUT OIL GELLED WITH 2% ALUMINUM MONOSTEARATE[a]

Time, Hr.	Number of Patients	Average Blood Level, Units/ml.	Patients With 0.03 or Higher, %
1	81	0.79	97
12	83	0.294	99
24	83	0.149	96
30	83	0.121	90
36	81	0.09	92
40	82	0.071	77
48	82	0.054	67

[a] Dose—1 cc. I.M.—300,000 units procaine penicillin G.

cles of procaine penicillin G in peanut oil gelled with 2% aluminum monostearate. This formulation is considerably better than the peanut oil alone and gives forty-eight hour blood levels.

Table XV represents the blood levels obtained in approximately 1,000 human subjects with a suspension of small particles (<5 μ) of procaine penicillin G in peanut oil gelled with 2% aluminum monostearate. This formulation is by far the best and gives ninety-six hour blood levels in 86% of the patients.

Figure 1 represents graphically the rabbit blood levels obtained with five repository penicillin formulations. It is evident that small particles of procaine penicillin G suspended in peanut oil gelled with 2% aluminum monostearate give the most delayed absorption.

Figure 2 represents graphically the blood levels obtained in human subjects with six repository penicillin formulations. Again it is evident that small particles of procaine penicillin G suspended in

TABLE XV—PENICILLIN SERUM CONCENTRATION IN HUMANS, PAM[a]—1008 PATIENTS

Time, Hr.	Number of Patients	Average Blood Level, Units/ml.	Patients With 0.03 or Higher %
1	369	0.292	99.5
2	98	0.323	96.9
3	15	0.767	100
12	186	0.136	96.7
24	795	0.231	98
36	164	0.081	91.4
48	809	0.133	96.7
72	980	0.092	94.1
96	977	0.065	85.9
120	464	0.033	61.6

[a] Dose—1 cc. I.M.—300,000 units of procaine penicillin G in peanut oil gelled with 2% aluminum monostearate.

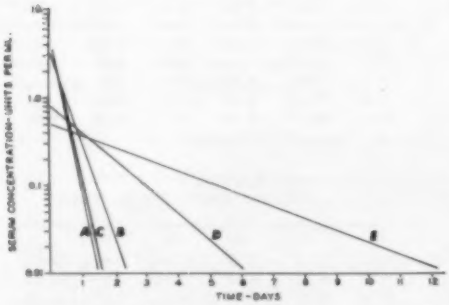

Fig. 1.—Penicillin levels in rabbit serum. Single injection—50,000 units per Kg. of suspensions containing 300,000 units per ml. *A*—Sodium penicillin G in peanut oil containing beeswax. *B*—Sodium penicillin G in aluminum stearate gel. *C*—Procaine penicillin G in peanut oil. *D*—Procaine penicillin G in aluminum stearate gel, large particles. *E*—Procaine penicillin G in aluminum stearate gel, small particles.

peanut oil gelled with 2% aluminum monostearate give the most delayed absorption.

SUMMARY

A considerable amount of data has been presented to show the effect of particle size and the nature of the vehicle on absorption of procaine penicillin G following intramuscular injections into rabbits. Serum concentrations in rabbits have been given to show the effect of some of the numerous variables influencing absorption.

The data obtained indicates that peanut oil or sesame oil gelled with an aluminum stearate gives a much more delayed absorption than either oil alone or oil plus beeswax. The data shows that, in an oil-aluminum stearate vehicle, small particles (less than 5 μ) are superior to large particles of procaine penicillin G in delaying absorption.

Serum concentrations in approximately 1,000 human subjects have been presented to show the superiority of small particles *versus* large particles of procaine penicillin G suspended in a vehicle of peanut oil or sesame oil gelled with 2 per cent aluminum monostearate.

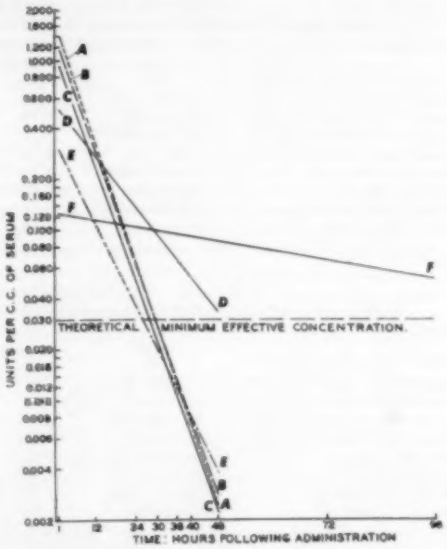

Fig. 2.—Average penicillin blood concentrations produced with single injections, 300,000 units each, of six repository penicillin preparations. *A*—Sodium penicillin G in peanut oil with 4.8% (w/v) white beeswax. *B*—Sodium penicillin G in peanut oil with 2% (w/v) aluminum monostearate. *C*—Procaine penicillin G in oil. *D*—Procaine penicillin G in peanut oil with 2% (w/v) aluminum monostearate (large particle). *E*—Aluminum penicillin in peanut oil with 2% (w/v) aluminum monostearate. *F*—Procaine penicillin G in peanut oil with 2% (w/v) aluminum monostearate (small particle).

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A Rheological Study of Procaine Penicillin G Depot Preparations*

By STEPHEN S. OBER, HUGH C. VINCENT, DANIEL E. SIMON, and KENNETH J. FREDERICK

Extent and type of thixotropic breakdown in aqueous, paste-like, procaine penicillin G preparations designed for intramuscular injection have been measured. The rheological properties of such suspensions are correlated with surface area and particle size distribution of the constituent powders. Certain thixotropic parameters of repository preparations are related to the nature of the depots formed in rabbit muscle and with product injectability and effectiveness in the human, as revealed by blood level data. A dilute gelatin gel has been found useful in forecasting and illustrating the nature of depots formed in muscle tissue.

PROLONGED therapeutic response from a single dose of an antibiotic preparation has long been the goal of pharmaceutical research. Many requirements have to be met if this is attempted in an intramuscular preparation. The sterile preparation has to be easily injectable. It must furnish high antibiotic levels in the blood for several days. And the product must be such that it can be made reproducibly and cheaply.

A number of methods have been used to make long acting formulations of penicillin for intramuscular use. The earliest was to suspend penicillin salts in a nonaqueous vehicle, as a means of retarding absorption. The second principle, which has found the greatest medical acceptance, is the use of penicillin salts which have relatively low solubility in water. Suspensions of procaine penicillin G in either aqueous or nonaqueous vehicles have been widely used to give therapeutic levels of the drug in the blood stream for twenty-four hours after a single dose.

Over eight years ago, it was discovered (1) that a high solids/water ratio, in aqueous suspensions of procaine penicillin G, was unusually effective in further delaying the absorption of the penicillin. The result was a product capable of yielding therapeutic blood levels for at least forty-eight hours after a single intramuscular injection. The effectiveness of this product depends upon both factors named above. The reduced solubility stems from the use of the procaine salt of penicillin G. The retarded absorption arises from the compact deposits readily formed within the muscle tissue by the very thick suspension.

Thick suspensions which will not flow except under pressure present many problems of manufacture and control. The present, detailed physical study of such suspensions was undertaken to solve these problems. How the various characteristics of the procaine penicillin powders affect the properties of the suspensions was studied first. Examined next was the relationship of suspension behavior to clinical effectiveness. This entailed a study of the nature of the drug depots formed in muscle tissue and a correlation of this information with clinical blood level data. Rheological characteristics were found to play a dominant role throughout all of this work.

EXPERIMENTAL

Preparation of Suspensions.—The procaine salt of penicillin G was used throughout this experiment. The aqueous vehicles were made up from the following chemicals: sodium citrate; methyl paraben; propyl paraben; polysorbate 80 (all U. S. P. XV), and water for injection.

The crystalline penicillin salts were broken down to the desired particle size range by either a milling or micronizing operation. The milled salts were prepared by passing coarse penicillin through a hammer mill. The micronized salts were prepared in an air reduction mill. Conditions of milling and micronizing were varied to give a range of particle sizes in each type of powder. Selected lots of milled and micronized powders were blended to yield the desired particle size distributions.

All finely divided powders were blended intimately with aqueous vehicles to give suspensions having per cent solids in the range of 40 to 70% (w/v). A typical aqueous vehicle was made up as follows: sodium citrate, 15.00 Gm.; polysorbate 80, 1.10 Gm.; methylparaben, 1.00 Gm.; propylparaben, 0.11 Gm.; and water, 545 ml. Minor changes in the vehicle formula were not found to be significant.

Specific Surface.—The surface areas of the penicillin salt powders were measured by means of the air permeability of a prepared powder bed. The choice of air permeability was guided by two factors: simplicity of the experimental technique, and

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The authors are indebted to Dr. E. C. Olson and Mr. D. Foreman for help in particle size distribution studies and suspension formulations, respectively, and also to Dr. R. W. Rinehart and Dr. R. W. Mattoon for much of the initial effort in the fields of specific surface and rheology.

anticipated closer correlation between the values obtained and the suspension rheological data.

Measurement of the penicillin powders followed A. S. T. M. Method C 204–55 for Portland cement (2), which method makes use of the Blaine Fineness Tester (3). Calibration of this instrument deviated from standard procedure to permit use of the Carmen-Kozeny equation in a modified form (4). The form selected took into account instrument geometry, spread in particle size distribution, and internal surface of the samples. This version of the Carmen-Kozeny equation is represented as follows:

$$Sw = \frac{K_s \cdot e^{3/2} \cdot t^{1/2}}{\rho \cdot \eta^{1/2} \cdot (1 - fe)}$$

where

Sw = specific surface of the powder, in sq. cm./Gm.

K_s = constant, evaluated through the use of National Bureau of Standards Portland cement standard, 114 g.

e = the fractional pore space of the packed powder bed,

$= \frac{1 - W}{\rho \cdot V}$ where W = weight of the powder sample, in Gm.; V = volume of the powder bed when compacted, in cc.; ρ = crystal density of sample, in Gm./cc.

t = the time, in sec., for the manometer meniscus to fall from the second line to the third line on the manometer tube.

η = viscosity of the air at the temperature of the determination, in poises.

f = factor related to instrument performance and powder sample characteristics.

Evaluation of f was carried out by running a single powder sample at a series of 4–5 plug weights. A plot was made of $t^{1/2}$ against $(1 - fe)/e^{3/2}$, letting $f = 1$. Progressively larger values of f were then assumed, until a value was found which gave a straight line through the origin satisfying the experimental points. On the basis of repeated trials it was found that this value of f could then be used with this particular instrument to handle all other powders of this material whose areas fell within the range ±25% of the area of the test sample and whose particle size distributions were all of the same general type. Whenever all of these conditions were not met, new values of f had to be determined to insure maximum reliability of the surface area data. Further details of this method will be published.

Values of specific surface of penicillin powders ranging from 3,000 sq. cm./Gm. to 100,000 sq. cm./Gm. have been measured in this fashion and used in the test suspensions to be described.

Particle Size Distribution.—Particle size distribution data were obtained for the various penicillin powders by means of an elaborate microscopic counting procedure. This procedure avoided some of the pitfalls commonly associated with microscope count data (5). Approximately 0.3% (w/v) dilute suspensions were prepared and counted with the microscope mounted under a Bausch and Lomb Model L photomicrographic camera. This arrangement served nicely to give the operator easy viewing as well as a ready means for permanent recording of selected fields. In this way the laborious job of counting could be staggered more effectively, and particle Brownian motion could be frozen. A surface active agent was added to the suspension to insure complete deagglomeration.

Since the samples to be counted often involved very broad particle size distribution ranges, it was necessary to employ a series of views at various magnifications and to normalize the resulting count data in the calculations. A standard engraved slide was used for purposes of calibration. Particles ranging in diameter from 0.5 to 200 μ were counted in this fashion.

To convert numerical count data to a relative weight per cent distribution requires knowledge of particle shape as a function of particle size. Thus, a particle shape distribution was determined also. Such observations and the appropriate numerical relationships were made and derived at the time of counting. The extremes found in the numerical distributions of these powders demanded these painstaking techniques. For example, the number of 0.5 to 10 μ size particles compared to the number of 90 to 100 μ size particles varied from 5,000 to 1 in the coarse powders to 5,000,000 to 1 in the fine powders.

To express the whole particle size distribution for each powder would be cumbersome, and the data would be difficult to follow. Further, when the complete data are given in this form, they tend to emphasize small differences in comparative distributions. This is particularly true at the particle size extremes, the very place where the counting method, regardless of technique, shows maximum error. Consequently, to avoid these difficulties the largest particle length in the first 10% of the small size end of the frequency spectrum, and the smallest particle length in the final 10% at the large size end, are reported.

The full details of the calculation and other aspects of the method will be published soon.

Rheology.—As already noted, the suspensions of the present study were high in solids/water ratio. Therefore, an instrument was needed which would give a continuous reading of viscosity over a very wide range of rate of shear. Such an instrument was found in the Hercules Hi-Shear Viscometer (6), a development of the Hercules Powder Co. for use on paper pulps.

The Hercules Hi-Shear Viscometer is a rotating bob instrument which provides a continuous plot of torque versus rate of shear over the rate of shear range from 0 to 4540 reciprocal seconds. The operation of this instrument has been described adequately by others (6, 7). It was modified only to the extent of making a new bob. This bob was machined to dimensions calculated to give a rate of shear of 2,000 reciprocal seconds at the maximum rotational speed of 1,050 r. p. m.

The thick penicillin salt suspensions used gave rheograms of the type shown in Fig. 6. Since the rate of change of shear on the Hercules is hand operated, the time allowed for decreasing the rate of shear from its maximum was held equal to the time used to increase it to this maximum (approximately thirty seconds each for both the up- and down-curves). These times were not critical. Minor deviations had little effect upon the rheograms,

except, possibly, for the area within the hysteresis-type loop of thixotropic breakdown.

Hypodermic Needle Plugging.—A special apparatus was designed and built to evaluate quantitatively the plugging tendencies of these thick suspensions when forced through standard hypodermic needles. This plug point apparatus consisted of a balance, one pan of which was coated with a rubber-like gum, a hypodermic syringe holder so arranged that this coated balance pan surface could be brought up flush against the hypodermic needle bevel, and a pressure system such that up to 200 pounds gas pressure could be applied to the hypodermic syringe. A schematic drawing of the instrument is shown in Fig. 1.

The plug point of a suspension was taken as the weight in Gm. sufficient to cause two out of three 20-gauge hypodermic needles to plug when subjected to 200 lbs./sq. in. pressure on the syringe plunger. Only loads up to 15 Gm. were used in this test. If 15 Gm. back pressure did not cause plugging, no further testing of plugging tendency was deemed necessary.

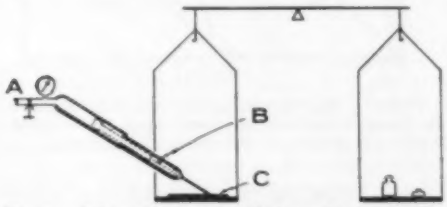

Fig. 1.—Schematic drawing of plug point apparatus. A.—To pressure tank. B—Suspension. C—Gum bed.

Gelatin Gels.—Injections of all test suspensions were made into 2% (w/v) gelatin gels to determine the nature of the deposits formed. The gelatin gels were prepared in the following manner: Two Gm. of gelatin was dissolved in 50 ml. of water at 100°. Fifty ml. of additional water was added, the mixture stirred and then poured into two-inch square glass containers. The gelatin was allowed to cool to 7° and set for eighteen to twenty-four hours. The resultant gelatin gels, when warmed to room temperature, were ready for injection.

The suspensions were injected into the gels using 20-gauge needles. Invariably, 600,000 units of penicillin per depot trial were injected. Photographs of the resulting depots were taken with a Bausch and Lomb Model L camera, employing the setup shown in Fig. 2.

Preparation of Suspensions for Clinical Trial.—Key combinations of milled and micronized procaine penicillin G powders, sterilized by treatment with ethylene oxide gas, were blended with sterile aqueous vehicle under aseptic conditions. These suspensions were then sent to Dr. Monroe J. Romansky, Medical Division, George Washington University, Washington, D. C., for clinical evaluation.

RESULTS AND DISCUSSION

Powder Characteristics.—Powder characteristics which were felt to control or contribute to suspension behavior included such physical properties as

Fig. 2.—Photographic setup for gel depots.

particle size, particle size distribution, particle shape, particle shape distribution, specific surface, density, and surface potential. It was quickly learned that some of these variables were not as significant as others. Therefore attention was directed toward the two most critical parameters, specific surface (*Sw*) and particle size distribution (PSD).

Specific surface or surface area per unit weight as determined by air permeability measures just those areas associated with the outside surfaces of the particles. The effect of cracks, crevices and small pores is set aside. This fact is very fortunate, since the behavior of procaine penicillin G powders in aqueous suspensions concerns only those areas accessible to water.

The distribution of individual particle sizes of a powder is also necessary to explain suspension behavior, even when the surface area of the powders is already known. This arises since a given specific surface can result from an infinite number of distributions, and yet each powder with a different distribution will lead to its own distinct suspension.

Typical PSD data for milled and micronized powders of procaine penicillin G are illustrated in Figs. 3 and 4. The distributions are presented as bar graphs made up of weight per cents for each 10 μ of particle length over the entire distribution range except for micronized powder (Fig. 4) where the 0–10 range was further subdivided. Also shown in

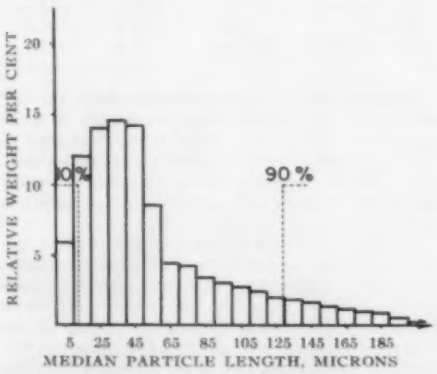

Fig. 3.—Particle size distribution of milled procaine penicillin G.

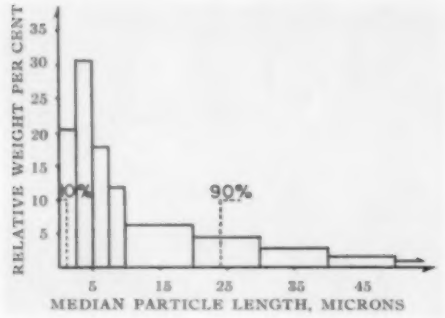

Fig. 4.—Particle size distribution of micronized procaine penicillin G.

these graphs are the particle lengths at the points of 10% and 90% by weight of the total powder sample, the first and last deciles. These figures are very useful in indicating the breadth of the distribution. However, it will be noted in this presentation that PSD data are far less significant than *Sw* data in correlating with suspension properties. This does not mean that PSD is unimportant. It merely reflects how difficult it is to obtain a PSD to a significant degree of accuracy even when great care is exercised.

The milled procaine penicillin G used in these experiments ranged in *Sw* from 3,000 to 9,000 sq. cm./Gm. with a corresponding PSD extending from 20 to 8 μ for the maximum particle length in the first decile to 175 to 85 μ minimum particle length in the last decile. The micronized powder ranged in *Sw* from 10,000 to 100,000 sq. cm./Gm. with a PSD from 7.5 to 1.0 μ in the first decile to 80 to 20 μ in the last decile. Table I gives two examples of *Sw* and PSD data for a milled and a micronized powder of procaine penicillin G used in this study. The full PSD for each of the two examples cited in Table I are given in Figs. 3 and 4.

TABLE I.—PROCAINE PENICILLIN G POWDER DATA

Powder Description	Specific Surface, sq. cm. per Gm.	Particle Size 1st Decile Maximum Length, microns	Distribution Last Decile Minimum Length, microns
Milled	5,360	13.0	129
Micronized	32,100	1.6	24

Rheology of Thick Suspensions.—Since the suspensions of the present study were high in solids (from 40 to 70%), they were thick, analogous to toothpaste, and would not flow except under pressure. When ejected from a hypodermic needle onto a flat surface, the suspensions had sufficient body and structure to stay coned.

A calculation showed that the rate of shear put on a fluid being ejected through a 20-gauge needle is, under average conditions, extremely high, *viz.*, above 2,000 reciprocal seconds. The Hercules Hi-Shear Viscometer provides a continuous reading of viscosity for rates of shear up to 4,540 reciprocal seconds. Various types of flow curves can be obtained on the Hercules Hi-Shear Viscometer and are illustrated in Fig. 5. If the sample is Newtonian, i.e., if the fluid possesses a viscosity which is independent of the rate of shear, the rheogram will appear as a straight line. In this case the reciprocal of the slope is directly proportional to the kinematic viscosity.

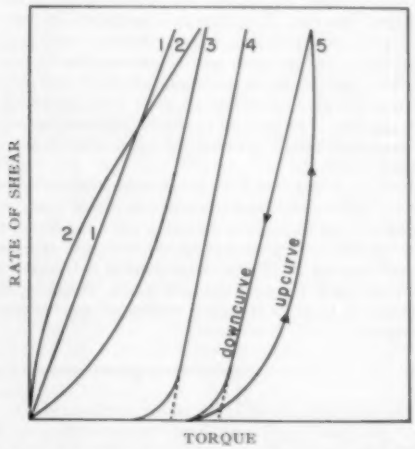

Fig. 5.—Typical rheograms from the Hercules Hi-Shear Viscometer 1—Newtonian, 2—Dilatant, 3—Pseudo-plastic, 4—Plastic, 5—Thixotropic.

Pastes, thick emulsions, ointments, and similar products, however, are non-Newtonian, the viscosity being dependent upon the rate of shear applied. For products such as these a wide variety of flow curves may be obtained. If the viscosity increases with increasing rate of shear, the system is said to be dilatant. Where the viscosity decreases with increasing rate of shear, the system is said to be plastic if the decrease is discontinuous, or pseudo-plastic if the decrease is continuous. If the material continues to decrease in viscosity at a constant rate of shear, it is said to be thixotropic. Thixotropy is shown in rheograms where the down-curve fails to coincide with the up-curve. The hysteresis-type loop so created is termed the area of thixotropic breakdown. Note that each of these qualities of a fluid is an independent property. As such, they can, and do, occur together in certain suspension systems.

The thick penicillin salt suspensions of this study gave rheograms of the type shown in Fig. 6. Several points of interest can be noted about the rheograms. The area under the hysteresis-type loop reflects the extent of thixotropic breakdown. Further, extrapolation of the down-curve to the torque axis provides a measure of the yield stress, shown as Y_1, Y_2, and Y_3. If, after the down-curve is completed, the rate of shear is promptly increased again, the down-curve is retraced going both up and down exactly as in the case of plastic flow. Similarly, if the rate of shear is held constant at some point such as A in the third rheogram, the torque will fall slowly to point C and there remain constant as long as no other conditions are altered. Thus, Y or yield stress values reflect fully sheared or stressed conditions of the system.

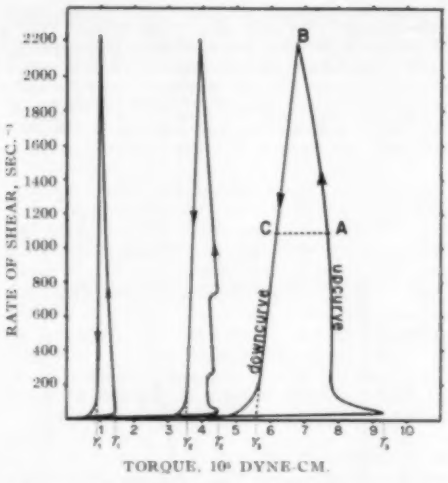

Fig. 6.—Typical rheograms of thick aqueous procaine penicillin G suspensions.

The most important aspect of the rheograms in Fig. 6, however, is denoted by points T_1, T_2, and T_3. These points of maximum torque on the initial up-curves reflect sharp breakdown points in the suspension structures at very low rates of shear. The T value proved to be the key to the entire problem of defining an injectable, long-acting product. It can be interpreted as a measure of the structure present in the suspension prior to any applied shear. The exact nature of this structure is not known, although the generalized picture presented by Fischer (8) appears to be acceptable.

Suspensions possessing T values were highly thixotropic. As the rate of shear increased on a fresh sample, which had not been subjected to recent shearing action, the suspensions showed virtually infinite viscosity. Upon further increase in rate of shear or continued application of a constant rate of shear, a maximum torque was developed. At this point the suspension commenced to flow. After the rate of shear had been applied for a definite time, an equilibrium condition was reached wherein the fluid behaved as a normal plastic system with just a small trace of dilatancy at high rates of shear.

Only a few minutes of rest was required for such suspensions to recapture their original structures. Thus, the initial up-curve of a rheogram was easily duplicated simply by removing the bob, releveling the sample in the cup, and repeating the determination.

It is felt that these rheograms explain why such thick suspensions are injectable and yet at the same time give prolonged therapeutic blood levels. The existence of a structural breakdown point, T, allows the thick paste to fluidize for passage through the hypodermic needle. On the other hand, the ability of the suspension to recapture quickly its structure probably accounts for the formation of a compact depot after intramuscular injection.

Correlation of Powder Characteristics with Suspension Data.—Data showing the effect of powder specific surface, Sw, upon the structural breakdown point, T, are given in Table II. These data include suspensions at three different penicillin levels. All the powders used were mixtures of micronized and milled grinds in a ratio of 3 to 1. This means their particle size distributions (PSD) were relatively broad and similar. These data are plotted in Fig. 7.

A series of suspensions was prepared next as part of a study on the effect of particle size distribution.

TABLE II.—EFFECT OF POWDER SPECIFIC SURFACE UPON SUSPENSION T VALUE AND PLUG POINT FOR POWDERS OF BROAD PARTICLE SIZE DISTRIBUTION[a]

Sample No.	Specific Surface (Sw), sq. cm./Gm.	Structural Breakdown Point (T), dyne-cm.	Plug Point, Gm.
	50% Solids		
1	15,100	123,000	3
2	21,400	215,000	3
3	24,000	355,000	10
4	27,500	425,000	>15
5	30,050	500,000	5
	55% Solids		
6	7,620	102,000	<3
7	15,040	150,000	7
8	18,750	250,000	10
9	20,200	411,000	15
10	21,550	500,000	10
11	24,000	578,000	>15
12	25,800	611,000	7
13	26,600	650,000	5
14	30,000	950,000	<3
	60% Solids		
15	12,750	180,000	3
16	14,950	280,000	10
17	16,200	422,000	12
18	17,950	575,000	10
19	21,300	745,000	5
20	24,000	1,000,000	5

[a] The PSD range was held from a maximum length of 1.5 μ for the 1st decile to a minimum length of 100 μ for the last decile. At least 75% of this range was covered in each case.

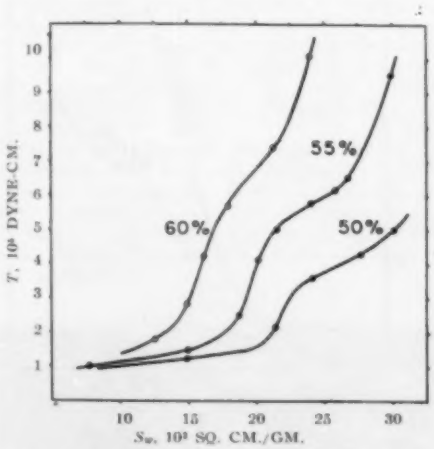

Fig. 7.—Influence of powder specific surface upon suspension structural breakdown point for powders with broad particle size distributions.

For this purpose special powders were prepared having relatively narrow distributions. The data for their suspensions are given in Table III.

TABLE III.—EFFECT OF POWDER SPECIFIC SURFACE UPON SUSPENSION *T* VALUE AND PLUG POINT FOR POWDERS OF NARROW PARTICLE SIZE DISTRIBUTIONS

Sample No.	PSD Deciles First max. 1 μ	PSD Deciles Last min. 1 μ	Specific Surface (*Sw*), sq. cm. per Gm.	Structural Breakdown Point, *T*, dyne-cm.	Plug Point, Gm.
21	12	105	<5,000	0	<3
22	8	90	10,800	50,000	3
23	2.5	39	17,000	292,000	5
24	1.9	28	19,100	573,000	5
25	1.9	26	22,000	1,020,000	<3

The data in Table III are plotted in Fig. 8, the resulting curve being labeled, "NARROW." Plotted alongside this curve are the data from Table II and Fig. 7 which correspond to the same per cent solids (55%). This latter curve is labeled, "BROAD." For purposes of comparison, the particle size data for a sample having a broad distribution, such as No. 12 in Table II, are 1.9 μ maximum length for the first decile and 80 μ minimum length for the last decile.

It will be seen that changes of this magnitude in PSD have a pronounced effect on suspension properties. The broad distribution curve shows a section where changes in *Sw* have far less influence on *T* than for the same region for the narrow PSD curve. This fact has practical importance, as will be discussed further in following sections of this report.

Next, studies of the effect of per cent solids upon suspension *T* value were made. Data are presented in Table IV and Fig. 9 where a typical powder was

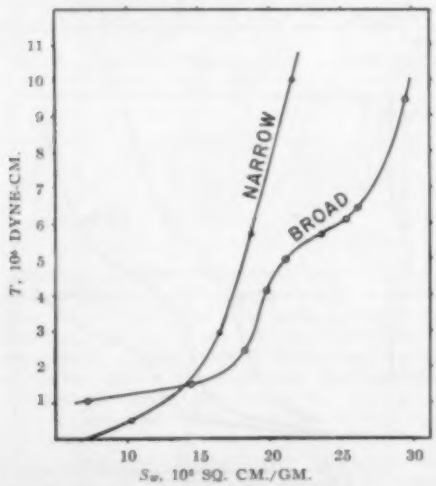

Fig. 8.—Influence of powder specific surface upon suspension structural breakdown point for powders with broad and with narrow particle size distributions. Both series run at constant per cent solids (55%).

TABLE IV.—EFFECT OF PER CENT SOLIDS UPON SUSPENSION *T* VALUE AND PLUG POINT FOR A POWDER OF BROAD PARTICLE SIZE DISTRIBUTION

Sample No.	Per Cent Solids	Structural Breakdown Point, *T*, dyne-cm.	Plug Point, Gm.
29	40	132,000	10
3	50	355,000	10
11	55	578,000	>15
27	58	780,000	5
20	60	1,000,000	5

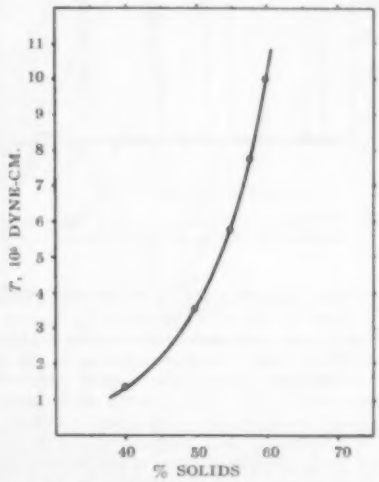

Fig. 9.—Influence of per cent solids upon suspension structural breakdown point for a powder of broad particle size distribution and constant specific surface.

used at varying weight per cent levels. Because this series was restricted to one powder, PSD and *Sw* were constant (1.9 μ maximum length for the first decile to 80 μ minimum length for the last decile for the PSD spread and 24,000 sq. cm./Gm. for *Sw*).

From the plots of the data given in Figs. 7 and 9, values of *Sw* and per cent solids for powders of broad PSD can be chosen which will give any desired suspension *T* value. This is done in Fig. 10 where the locus of four different *T* values for many combinations of *Sw* and per cent solids are shown.

Injectability of Suspensions.—The question of possible plugging of hypodermic needles becomes important whenever thick suspensions are to be injected into muscle tissue. Those who have handled paste-like suspensions know that if a finger is placed over the bevel of a standard hypodermic needle and pressure is applied to the syringe plunger, some of the samples will plug. In such instances, even after the finger is removed from the needle tip, the suspension can no longer be ejected from the syringe. An instrument previously described was devised to measure a suspension's plugging tendency.

Although plug point is an easy measurement to make, the results are subject to wide variations. Repeated determinations on the same or new

samples can give a range of values from low plug weights to the highest reported of greater than 15 Gm. For this reason, wherever possible, plug point data are taken as the average of a number of separate determinations. Any suspension with an average plug weight of 5 Gm. or more has been found through extensive clinical trials to be easy to handle and inject.

In Tables II–IV, suspensions which have a high T value, approaching 1,000,000 dyne-cm. due either to a high Sw or a high % solids, or both, can be seen to have poor plug points. Similarly in the region of low T value where combinations of Sw and % solids are much more restricted, the resulting products tend to plug easily. This region of poor operation is best eliminated by limiting specific surface to values in excess of about 10,000 sq. cm./Gm. In sharp contrast, suspensions having T values in the range of 150,000 to 750,000 dyne-cm., and Sw values in excess of 10,000 sq. cm./Gm. show far less tendency to plug.

On the basis of these data, the line AB in Fig. 10 represents a maximum limit imposed by the requirement of easy injection. This limit occurs at approximately a T value of 1,000,000 dyne-cm. Similarly, the line BC represents a minimum limit also imposed by injectability. This limit approximates powder specific surface values of 10,000 sq. cm./Gm. Suspensions which are made up of powders whose specific surface exceeds 10,000 sq. cm./Gm., and which possess T values below 1,000,000 dyne-cm., will handle and inject easily.

The area of practical operation for powders of wide PSD is partially enclosed by the two lines AB and BC. If the PSD is not widespread (Table III), the plug point data call for a lower maximum T and a higher minimum Sw. This shrinks the operational area within AB and BC considerably.

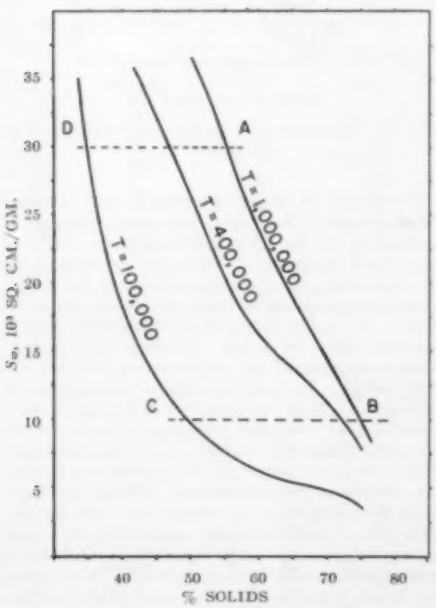

Fig. 10.—Locus of given suspension T values for various combinations of S_w and per cent solids for powders with broad PSD.

Correlation of Rheological Properties with Depot Formation.—The clinical effectiveness of repository preparations of penicillin is usually judged by blood level data, either in humans or in test animals. The large number of suspensions involved in the present study precluded such extensive data. For this reason a method was sought for measuring the ability of the test suspensions to form depots upon injection into muscle.

Initially test suspensions were injected into dead beef muscle. Some injected test suspensions which had substantial T values did indeed form rounded and compact depots. However, the tests were difficult to evaluate and in some cases were not reproducible.

Therefore selected trial suspensions were injected into rabbits, and after varying periods of time, these animals were sacrificed. The muscle tissue was opened, and if any depot was found, it was extracted and photographed. In Fig. 11 three depots extracted from rabbit muscle twenty-four hours after injection are illustrated. The numbers correspond to suspensions of varying but known T values (as listed in Table VI). Note that No. 6, one which has a T value of 100,000 dyne-cm., gave a depot which was slightly elongated and not as compact as the depots from Nos. 11 and 12. Suspensions which possessed no T value at all, even though they were paste-like and contained high penicillin unitage, gave no depot at all. In fact after twenty-four hours no trace of the procaine penicillin G could be found.

While the rabbit studies indicated clearly that the T value was related directly to the nature of the depot formed upon injection into muscle, it was even impractical here to test all of the many hundred suspensions by this means. A search for an *in vitro* technique of depot formation evaluation was rewarded when 2% gelatin gels were injected with 600,000 units of penicillin G per trial. The resulting spherical depots formed in gelatin by suspensions possessing T values in excess of 100,000 dyne-cm. resembled closely those extracted from the sacrificed rabbits. Since the gel was transparent, depots were not disrupted or altered for the sake of viewing. Furthermore they could be photographed for a permanent record.

Figures 12–15 illustrate the range of gelatin gel depots observed in this study. Suspensions, such as examples No. 21 and No. 28 which gave no measurable T values, did not form compact deposits. Instead the particles spread widely throughout the gels. Figures 12 and 13 show top and side views of the fan-shaped injections.

Fig. 11.—Depots extracted from rabbits. Reading from left to right, depots from suspension samples 6, 12, and 11.

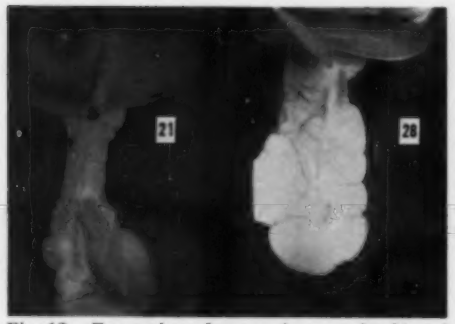

Fig. 12.—Front view of suspension samples 21 and 28 injected into gelatin.

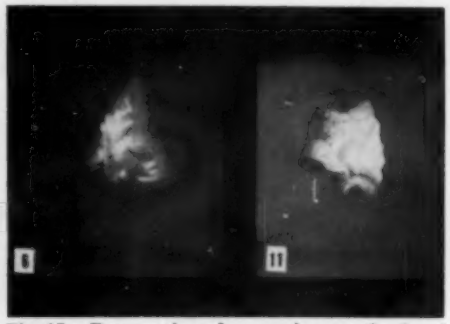

Fig. 15.—Bottom view of suspension samples 6 and 11 injected into gelatin.

Fig. 13.—Side view of suspension samples 21 and 28 injected into gelatin.

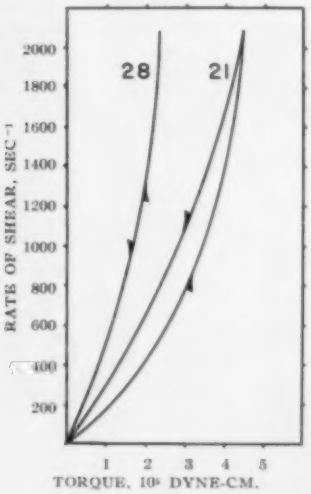

Fig. 16.—Rheograms of suspension samples 21 and 28.

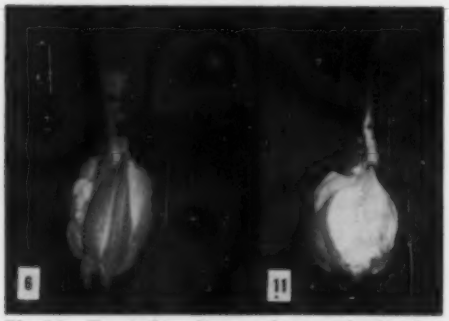

Fig. 14.—Front view of suspension samples 6 and 11 injected into gelatin.

In contrast, Figs. 14 and 15 show the compact, round depots formed by two systems which possess measurable T values. Suspension No. 6 has a minimum T value of 100,000 dyne-cm., while No. 11 is representative of many of the suspensions tested clinically. Number 11 has a T value of 578,000 dyne-cm. The rheograms of these two suspensions are shown in Figs. 16 and 17.

The gelatin depot test was used on a large number of suspensions. From this accumulation of data the optimum range for each of the many suspension variables was thoroughly explored. The results were consistent and left little doubt that the formation of a compact depot requires a suspension with a measurable structural breakdown point (T value).

Correlation of Depot Formation with Clinical Effectiveness.—The depot formation indicates a mechanism for the prolonged therapeutic action of these thick suspensions. The compact depot resists the dissolving pull of the tissue fluids by the simple means of presenting a minimum surface to these fluids. To substantiate this correlation, clinical penicillin blood level data on selected key suspensions were obtained and are presented in Table V.

In order to evaluate the clinical performance of the various test lots on some sort of a rating basis, a value of 1 was assigned arbitrarily to each step or dilution increment in the blood level determination. Then, for the forty-eight and seventy-two hour data, the deviations from the overall medians for these times were added up algebraically for each sample. This sum of unit variations appears as the rating in the last column. Any variation beyond $\pm 1^1/_2$ units was considered to be significant. This rating was based on the median of all the samples, excepting Nos. 21 and 28.

In the last group of test lots in Table V all of the suspensions had T values in excess of 100,000 dyne-

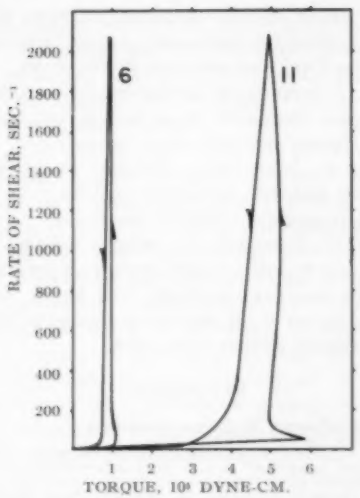

Fig. 17.—Rheograms of suspension samples 6 and 11.

cm., and all gave excellent penicillin blood levels. Inasmuch as 0.03 unit of penicillin G per cc. is considered to be the minimum therapeutic response, it can be seen that even at the seventy-two and ninety-six hour points these particular examples are most satisfactory repository products.

The physical properties of the clinical samples are summarized in Table VI.

Area of Practical Operation Including Repository Action.—The clinical blood level data confirm the significance of depot formation and, in turn, the importance of measurable T values. While the suspensions chosen for clinical trial did not include any with T values in excess of 650,000 dyne-cm., all other evidence indicates such suspensions would be equally effective.

A lower limit of 100,000 dyne-cm. for suspension T values is apparent from consideration of the borderline status of No. 6. The rating of No. 6 is subnormal, even though it still gives an acceptable response at seventy-two hours in comparison to Nos. 21 and 28. The latter two are just minimal.

The area of practical operation can now be fully defined. As noted previously, to be injectable the product must be made up of powders whose specific surface values exceed 10,000 sq. cm./Gm. Also, the T value of the suspension must be less than 1,000,000 dyne-cm. These conclusions led to the limits expressed as lines AB and BC (Fig. 10). In addition, the clinical trials provide another limit to this area. To be repository in action the suspension must have an appreciable T value. This limit has been shown to be about 100,000 dyne-cm., and so line CD can now be drawn.

At this point the area of practical operation appears as being open-ended. Theoretically, this is true. However, the grinding and handling of powders of 30,000 sq. cm./Gm. or higher specific surface becomes increasingly difficult. Consequently, there is a practical limit in this direction too, which is represented as the line DA in Fig. 10. The area of practical operation is now fully enclosed.

TABLE V.—CLINICAL BLOOD LEVEL DATA ON SUSPENSIONS OF PROCAINE PENICILLIN G

Sample No.	No. of Patients	Penicillin Blood Level Medians[a] in Units/cc. 4 hr.	24 hr.	48 hr.	72 hr.	96 hr.	Rating
28	12	2.5	2.5	0.03	0.03	0.03	−7½
21	13	5.0	2.5	0.47	0.03	0.03	−3
6	10	0.31	0.31	0.16	0.16	..	−2½
29	15	0.94	0.62	0.31	0.16	..	−1½
30	10	0.94	0.47	0.31	0.23	..	−1
12	10	7.5	2.5	0.62	0.23	0.16	0
11	12	10.0	2.5	0.62	0.31	0.08	½
9	12	2.5	1.25	0.62	0.62	0.06	1½
24	12	1.25	0.47	0.62	0.62	..	1½
Median[b]		1.25	0.62	0.62	0.23	0.08	..

[a] Clinical penicillin blood data by Dr. M. J. Romansky of George Washington University Hospital; 600,000 units of penicillin per dose for each patient.
[b] Excluding samples Nos. 21 and 28.

TABLE VI.—SUMMARY DATA

Sample No.	Ratio Micronized to Milled PGP	PSD —Deciles— First Max. 1, μ	Last Min. 1, μ	Specific Surface, sq. cm. per Gm.	Per Cent Solids	Struct. Breakdown Point, T, dyne-cm.	Gelatin Depot Description	Plug Point, Gm.	Clinical Rating
28	0/100	7.3	112	>5,000	55	0	Flat and fanned	<3	−7½
21	0/100	12	105	>5,000	55	0	Flat and fanned	<3	−3
6	75/25	8.5	89	7,620	55	102,000	Oval	<3	−2½
29	75/25	2.3	79	24,000	40	132,000	Slightly oval	10	−1½
30	60/40	3.2	95	25,000	55	414,000	Spherical	>15	−1
12	75/25	2.0	85	25,800	55	611,000	Spherical	7	0
11	75/25	2.3	79	24,000	55	578,000	Spherical	>15	½
9	75/25	2.5	90	20,200	55	411,000	Spherical	15	1½
24	100/0	1.9	28	19,100	55	573,000	Spherical	5	1½

Figure 10, it will be remembered, represents only powders (or powder mixtures) having wide and even distributions of particle size. Similar charts can be prepared for powders with different PSD. However, the narrower the distribution, the smaller the area of satisfactory operation becomes.

SUMMARY

1. Thick aqueous suspensions of 40 to 70% by weight of procaine penicillin G are shown to be suitable for intramuscular injection and to have a repository effect.

2. The most significant physical parameters of such suspensions are shown to be specific surface (*Sw*) and relative weight particle size distribution (PSD) of the powder, rheological structural breakdown point (*T*), and per cent solids of the suspension.

3. These physical properties are correlated with suspension effectiveness by means of injection into gelatin gels and rabbits, and clinical blood level data.

4. To be readily injectable, a thick aqueous suspension of procaine penicillin G must have been made from a powder having an *Sw* in excess of 10,000 sq. cm./Gm. and a relatively broad distribution of crystal or crystal fragment sizes. Furthermore, the suspension must not be so thick that the *T* value exceeds 1,000,000 dyne-cm.

5. To insure a clinical response in excess of the minimum therapeutic blood level of 0.03 unit of penicillin/cc. for forty-eight, seventy-two, and ninety-six hours after injection of a single 600,000 unit dose, the suspensions must show *T* values in excess of 100,000 dyne-cm.

6. The formation of a compact, spherical depot in the muscle accounts for the prolonged therapeutic response observed. The highly thixotropic nature of the suspension appears to be responsible for its ease of injection.

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Principles in the Spectrophotometric Determination of Propoxycaine in the Presence of Procaine*

By EDWARD G. FELDMANN, WALTER MAHLER,† and HENRY M. KOEHLER

A spectrophotometric method for the determination of propoxycaine in the presence of procaine has been developed. Aqueous mixtures of these two local anesthetics in the form of their hydrochloride salts, which are commercially marketed, may be assayed for propoxycaine by an extension of principles developed in a study of a series of simple model compounds. The use of 2 *N* hydrochloric acid as a solvent permits the direct determination of propoxycaine with only a minimum of interference from comparable concentrations of procaine which may be present.

Since local anesthetic compounds of the procaine or *p*-aminobenzoate ester group display a high degree of absorption in the range of 250–310 mμ, ultraviolet spectroscopy has provided a convenient means for the assay of most of these compounds. Generally such assay methods are relatively free of complicating factors, even when applied to dosage forms of the local anesthetics, since other materials present either (*a*) do not display absorption in this area, (*b*) are diluted to the point where their absorption is negligible, or (*c*) may be conveniently removed by chemical separation procedures by virtue of gross differences in their physical and chemical properties from those of the local anesthetic compounds.

However, a number of local anesthetic preparations, which contain a mixture of two anesthetic compounds, are currently being marketed to the dental profession (1). One such product

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incorporates a mixture of 2% 2′-diethylaminoethyl 4-aminobenzoate hydrochloride (procaine hydrochloride) and 0.4% 2′-diethylaminoethyl 2-propoxy-4-aminobenzoate hydrochloride (propoxycaine hydrochloride).[1]

Propoxycaine hydrochloride can be conveniently assayed alone by simple ultraviolet methods (2) or by spectrophotometric titration with nitrous acid (3). The presence of procaine hydrochloride will, however, interfere with both of these methods, as well as most classical methods such as modified acid-base titrations which are employed in the U. S. P. assay for procaine hydrochloride (4), and the N. F. assay for lidocaine hydrochloride (5). The only record of a procedure for the determination of propoxycaine in the presence of procaine (6) is a modification of the Zeisel hydriodic acid cleavage method for the determination of alkoxy groups as described by Elek (7). This method generally gives satisfactory results, but it requires very careful technique and is long and tedious. Because of these factors and the additional consideration that the method requires relatively large samples, it is not well suited to routine use.

In view of the simplicity and general desirability of the ultraviolet absorption methods, the possibility of a modified direct spectrophotometric method was investigated. The absorption spectrum above 250 mμ of procaine monohydrochloride can be primarily attributed to the effect of the aromatic primary amine group, while that of propoxycaine monohydrochloride is primarily due to the combined effect of the aromatic amine group plus the aromatic alkoxy group. The absorption spectra of these two compounds in aqueous solution are shown in Fig. 1. Absorption below 300 mμ can be attributed almost entirely to the influence of the aromatic amine, and in small part to the ester group, while absorption in the neighborhood of 300 mμ and above can be attributed to the effect of the alkoxy radical.

Since the tertiary alkyl amine is by far the more basic amine in both of these compounds, it follows that the monohydrochlorides are formed by salting the alkyl amine, leaving the aromatic amine with its free electron pair. Resonance of this electron pair with the aromatic ring results in the high degree of absorption common to aromatic amines. Similarly, resonance of an electron pair of an alkoxy oxygen with the ring is also responsible for long wavelength absorption. Several investigators (8, 9), among others, have shown that the absorption due to an aromatic amine is strongly, if not completely, depressed in strong acid solution.[2] This is because the electron pair is firmly bound by salt formation, and resonance with the ring is no longer possible. Since phenolic groups or alkoxy groups are weaker bases than water, binding of an oxygen electron pair would occur only in very strong acid solution, such as in concentrated sulfuric acid. Hence in aqueous acid solution the alkoxy group would be expected to continue to display significant absorption. It follows, therefore, that in an aqueous acid system which is sufficiently acid to convert the aromatic amines to their respective salts, a mixture of an aromatic amine and an alkoxy aromatic amine should exhibit total absorption which would essentially be that of the corresponding aromatic alkoxy compound.

A series of simple model compounds was employed to establish this general premise. The generalizations drawn from the study of

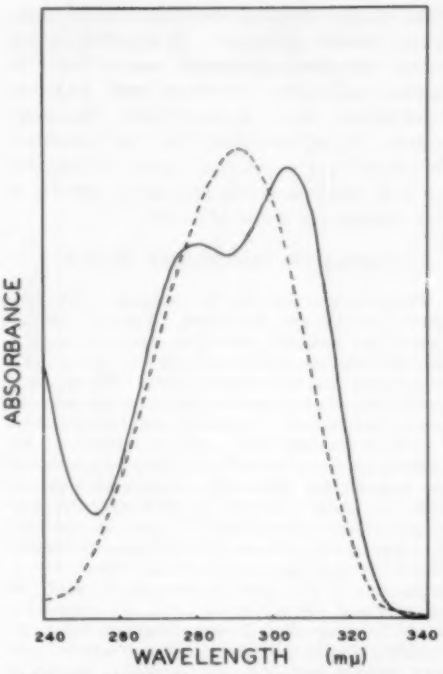


Fig. 1.—Absorbance curves of aqueous solutions; - - - - procaine hydrochloride, ——— propoxycaine hydrochloride.

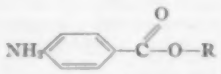
[1] The brand name of Cook-Waite Laboratories, Subsidiary of Sterling Drug, Inc., for procaine hydrochloride is Novocain, and for propoxycaine hydrochloride is Ravocaine hydrochloride.

[2] Pratt (3) notes in his publication that absorption of both procaine and propoxycaine diminishes in acid solution, but he did not attempt to exploit this effect for assay purposes.

these model compounds were then applied to the specific system of interest. As expected, it was found that the experimental results with the specific compounds correlated well with the experimental data obtained from the model system. It is concluded that this procedure therefore offers a potential means of assaying not only this system but also other mixtures of this same general chemical nature.

CHOICE OF THE MODEL SYSTEM

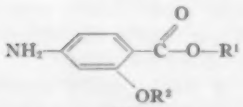
The structural formulas for procaine (I) and propoxycaine (II) are illustrated. Kumler (10) has shown that aromatic carboxylic esters of nonchromophoric alcohols have essentially the same qualitative spectra, and only a very slightly different quantitative absorbance than the corresponding aromatic free carboxylic acid. Robertson and his co-workers (11) have demonstrated a similar relationship between phenols and their alkoxy derivatives, although the quantitative differences are somewhat greater with this group. In view of these considerations, ideal model compounds would be those in which the R groups of both procaine and propoxycaine are substituted by hydrogen atoms, giving, respectively, *p*-aminobenzoic acid and *p*-aminosalicylic acid. In aqueous acid solution, these latter two compounds would be expected to display absorption closely resembling that of the corresponding nonamino analogs, benzoic acid, and salicylic acid. As seen in Figs. 2 and 3 this is indeed the case; that is, absorption due to the aromatic amine group, which is very strong in simple aqueous solution (Fig. 2), disappears in aqueous acid solution (Fig. 3). The absorption spectra of benzoic acid and salicylic acid are included in both figures therefore to show that while there are great differences from the absorption of the amino analogs in aqueous solution, the absorption spectra of the amine salts are very similar to their nonamino counterparts in acid solution. Furthermore, it can be seen that, qualitatively, very negligible differences occur in the absorption of both benzoic acid and salicylic acid when employing the acid as the solvent.



I

p-Aminobenzoic Acid
R = —H

Procaine
R = $-CH_2CH_2N(C_2H_5)_2$



II

p-Aminosalicylic Acid
R^1 = —H; R^2 = —H

Propoxycaine
$R^1 = -CH_2CH_2N(C_2H_5)_2$
$R^2 = -CH_2CH_2CH_3$

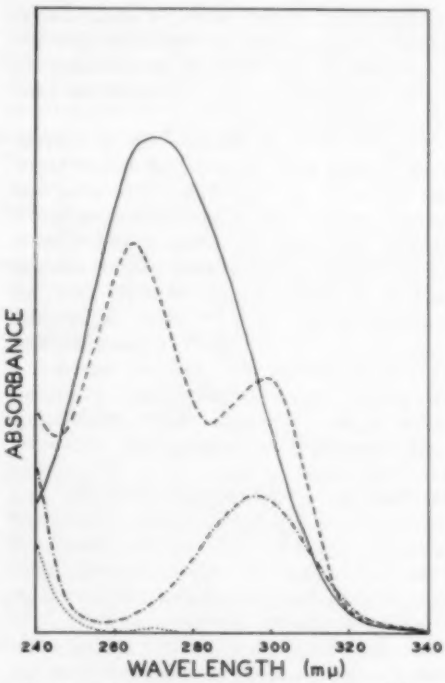


Fig. 2.—Absorbance curves of aqueous solutions; - - - - *p*-aminosalicylic acid, ——— *p*-aminobenzoic acid, - - - - - - - - salicylic acid, ... benzoic acid.

APPLICATION TO THE PROCAINE–PROPOXYCAINE SYSTEM

Similar results were seen when the absorption spectra were taken for procaine and propoxycaine in acid solution. As seen in Fig. 4, absorption at 290 mμ and below, attributed to the amine-ring resonance, has disappeared except for residual absorption from the ester radical, while propoxycaine absorption in the 300 mμ area is still significant. In fact, procaine absorption at 300 mμ is negligible, so that in a solution containing both materials, total absorption at 300 mμ can be attributed to propoxycaine alone. This wavelength is chosen for purposes of determination because, while the absorption of propoxycaine is essentially the same as at its peak at 296 mμ, the ratio of propoxycaine absorption to total absorption is closer to 1.0. Therefore, error due to ignoring the presence of procaine is made even less significant. After establishing experimentally that both anesthetic compounds follow Beer's law at this wavelength in this solvent system, mathematical calculations based on the experimentally determined absorption values show that an error of slightly less than 0.5% results when the presence of an equal concentration of procaine in the propoxycaine sample is ignored. Even in the commercial solutions, where the procaine to propoxycaine ratio is 5:1, this error is only 2.2%. An error of this size is of no significance in routine or control measurements, and is within the range of the expected in-

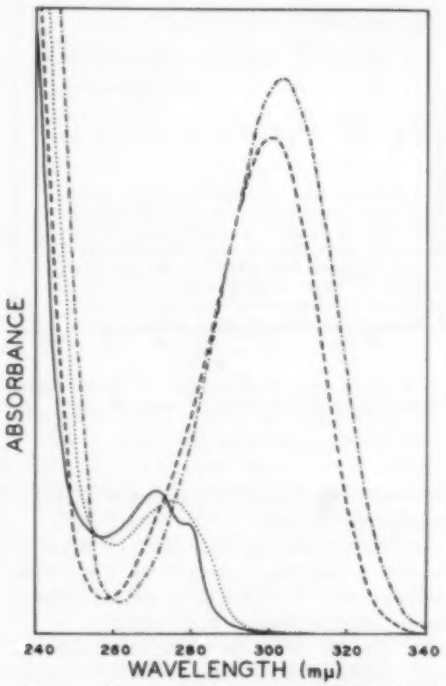


Fig. 3.—Absorbance curves of 2 *N* hydrochloric acid solutions; - - - - *p*-aminosalicylic acid, ——— *p*-aminobenzoic acid, - - - - - - - - - salicylic acid, ... benzoic acid.

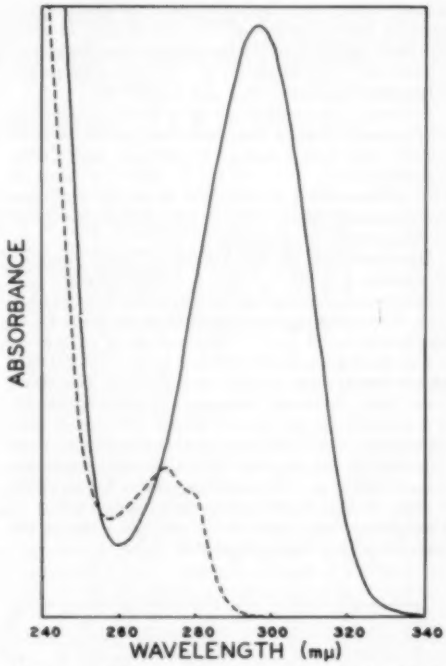


Fig. 4.—Absorbance curves of 2 *N* hydrochloric acid solutions; - - - - procaine hydrochloride, ——— propoxycaine hydrochloride.

strument error or variability (12, 13). If desired, it is possible to reduce further this small source of error, since it is proportional to the procaine concentration present. The procaine concentration is significant only when it is present in overwhelming concentrations, in which case it can be estimated as equivalent to the concentration of the total anesthetic. Total anesthetic concentration can be conveniently determined by the general method of Hirt and co-workers (14) using aqueous solutions and measuring absorption at the isoabsorptive point of 305 mμ (2).

In order to choose an acid concentration which would be suitable, pKa values for the two local anesthetics were determined. Acid concentration is significant because conversion of the aromatic amines to the respective salt forms is, of course, pH dependent. The method used for determining the ionization constants was that of Flexser and coworkers (15), in which absorption is measured as a function of pH. Using this procedure, the pKa for the aromatic amine of procaine was found to be 2.1, which agrees well with the literature value of 2.01 (16). For the aromatic amine of propoxycaine the pKa was found to be 1.9. On the basis of these values there is better than 99% conversion to the salt forms for both compounds in a 2*N* solution of a strong mineral acid. Hydrochloric acid was chosen as this acid since the compounds are employed in dosage forms as the monohydrochloride salts.

EXPERIMENTAL

Equipment and Materials.—Absorption data were taken using a Beckman DU spectrophotometer with photomultiplier attachment, and silica cells of 1.00 cm. light path. Measurements of pH were made using a Beckman model G pH meter. A sample of propoxycaine hydrochloride, m. p. 148–150° after recrystallization from ethanol-ethyl acetate, was generously provided by Cook-Waite Laboratories. All other chemicals used are commercially available; the purest grades available of each of the solid materials were recrystallized from suitable solvent systems.

Preparation of Solutions.—The spectra in Fig. 1 were determined from solutions prepared from accurately weighed samples of procaine hydrochloride and propoxycaine hydrochloride which were appropriately diluted with water to concentrations of 4×10^{-5} *M*. The spectra seen in Fig. 2 were taken on similarly prepared aqueous solutions of benzoic acid, salicylic acid, *p*-aminobenzoic acid, and *p*-aminosalicylic acid which were made up to final concentrations of 5×10^{-5} *M*. Solutions used to obtain data for the preparation of Fig. 3 were made up by diluting 2.0 ml. of aqueous 1×10^{-2} *M* solutions of the four compounds with 20 ml. concentrated hydrochloric acid and then making the solutions up to 100 ml. with water, giving final concentrations of 2×10^{-4} *M*. The resulting solutions are approximately 2 *N* with respect to hydrochloric acid.

The spectra in Fig. 4 were taken on solutions of procaine hydrochloride and propoxycaine hydro-

chloride which were similarly prepared giving aqueous 1×10^{-3} *M* solutions; 2.0 ml. of each of these was then diluted with 20 ml. of concentrated hydrochloric acid and made up to 100 ml. with water, giving final concentrations of 2×10^{-4} *M*.

Solutions for verification of a direct proportion relationship between absorbance and concentration (Beer's law) were prepared by making appropriate graded dilutions of each of the 2×10^{-4} *M* solutions with additional 2*N* hydrochloric acid. In each case the absorption values were, as expected, proportional to the concentration present.

Determination of pK Values.—Fifteen aliquots of aqueous 4×10^{-4} *M* procaine hydrochloride and propoxycaine hydrochloride solutions were diluted with varying concentrations of hydrochloric acid to give a series of 4×10^{-5} *M* solutions of each compound having a pH range from 0 to 6. The pH and absorbance of each solution in each of the two series were then accurately determined experimentally, and a curve of absorbance *versus* pH was drawn. Absorption values of the procaine solutions were measured at 290 mμ and of the propoxycaine solutions at 302 mμ. The resulting curve for procaine is seen in Fig. 5 and for propoxycaine in Fig. 6. The pKa values were taken as the pH value at the midpoint of the sharp slope of each curve.

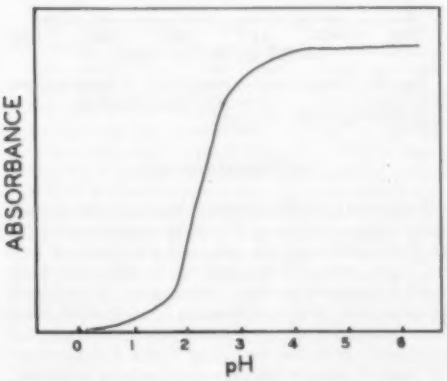


Fig. 5.—Curve of absorbance of procaine hydrochloride at 290 mμ in solutions of graded pH.

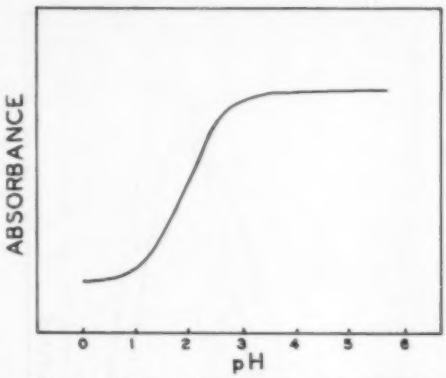


Fig. 6.—Curve of absorbance of propoxycaine hydrochloride at 302 mμ in solutions of graded pH.

SUMMARY

1. Generalized principles correlating absorption characteristics with solution pH have been elucidated for a family of compounds related to *p*-aminosalicylic acid.

2. These principles have been employed to develop a convenient method for the routine determination of propoxycaine hydrochloride in the presence of procaine hydrochloride.

3. The pK values for the aromatic amine of each compound have been determined and are reported.

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Correlation of *in vivo* with *in vitro* Disintegration Times of Enteric Coated Tablets*

By JOHN G. WAGNER, WILLIAM VELDKAMP, and STUART LONG

The stomach retention and intestinal disintegration times of six lots of enteric coated tablets were determined roentgenographically in the dog. The resistance of the coatings to simulated gastric fluid U. S. P. and disintegration times of the whole enteric coated tablets and of the subcoated tablets in artificial intestinal fluid, pH 6.9, were determined. Graphs and equations are presented which express the observed relationship between the average intestinal disintegration time in the dog and the average disintegration time of the whole enteric coated tablet or of the enteric coating only in artificial intestinal fluid. All data were analyzed statistically.

THE LITERATURE concerning the coating of tablets, pills, and capsules including the methods which have been used to test enteric coatings was recently reviewed by Wagner (1). Since that review a number of publications concerning the testing of enteric coated tablets have appeared (2–8).

Kanig (9) in discussing enteric coatings, stated: "It is obvious that it is difficult, if not impossible, for human subjects to be utilized in a repeated quality control procedure of testing during large-scale production. For this reason it is of added importance that an established and standardized *in vitro* testing method be utilized as an additional check in the manufacturing laboratory."

A satisfactory *in vitro* test method should give results which can be correlated with results obtained *in vivo*. The purpose of this study was twofold: (a) to investigate the dog as a test animal to determine stomach retention times and intestinal disintegration times of different enteric coated tablets; and (b) to correlate intestinal disintegration time in the dog with disintegration time obtained by an *in vitro* method. Although the human being and the rat have been used in roentgenographic studies with enteric coated tablets (1) we are unaware of any published information that the dog has been utilized in such studies. The dog is susceptible to laboratory controls and thus provides a possible method for the screening of enteric coatings and for determining the effects of various storage conditions on *in vivo* disintegration time. Preliminary evidence indicates that the results obtained in the dog are similar in many respects to results obtained in human subjects and such data will be the subject of a future communication.

* Received November 20, 1957, from The Upjohn Company, Kalamazoo, Mich.

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EXPERIMENTAL

***In Vivo* Studies.**—Dogs trained to lie quietly on the X-ray table were used. For those studies directed toward evaluating the stability of the coatings in the stomach the dog was fed thirty minutes prior to the administration of four tablets. Under these conditions gastric elimination exceeded seven and one-half hours. For the balance of the studies (Table I) the dogs were administered four enteric coated tablets and, in all except two cases, 50 cc. of 0.1 *N* hydrochloric acid, following an overnight fast. X-ray photographs were taken forty-five minutes following administration of the tablets and at intervals of fifteen minutes or less thereafter. Each figure entered under a dog number in Table I represents as accurate an estimate as possible of the average retention time in the stomach or the average intestinal disintegration time of the four enteric coated tablets of each run.

Compressed and Subcoated Tablets.—Compressed tablets containing 3 grains of barium sulfate were used in all studies. They were subcoated prior to enteric coating. The properties of the compressed and subcoated tablets are shown in Table II.

Enteric Coated Tablets.—The subcoated tablets were coated with five different enteric compositions by conventional pan coating techniques. The nature of these coatings will be described in a subsequent publication. A sample of one of the lots of tablets (lot II) was stored in a flint glass bottle at 47° for one month and this sample is designated II* in Table III. The properties of the enteric coated tablets are outlined in Table III.

***In Vitro* Disintegration Tests.**—The investigations described in this publication were carried out before simulated intestinal fluid U. S. P. XV (10) became official. The artificial intestinal fluid, pH 6.9, used in this study had the following composition: pancreatin U. S. P., 10.0 Gm.; ox bile extract U. S. P., 4.0 Gm.; M/5 potassium biphosphate, 250.0 ml.; M/5 sodium hydroxide, 140.0 ml. (or sufficient); and deionized water, *q. s.* to 1000.0 ml.

Disintegration tests were carried out on the enteric coated tablets by first subjecting the tablets to simulated gastric fluid U. S. P. (pH 1.2) at 37 ± 2°, for a period of two hours. All tablets tested were unaffected by this treatment. The tablets were then transferred to the artificial intestinal fluid,

TABLE I.—PERIOD OF RETENTION IN STOMACH (S) OF STARVED DOGS AND INTESTINAL DISSOLUTION TIME (T) OF ENTERIC COATED TABLETS, TIME IN MINUTES

	Dog									
	No. 1		No. 2		No. 3		No. 4		Averages	
Tablets	S	T	S	T	S	T	S	T	S	T
I	146	25	67	37	102	32	...	...	98[b]	32.2[b]
	87	33	99	41	86	25	...	...	(113)[c]	(34.1)[c]
II	90	50	45	45	70	41	...	...	73[b]	45.2[b]
	90	45	65	40	75	50	...	...	(88)[c]	(47.1)[c]
III	100	67	110	45	80	55	...	...	105[b]	54.0[b]
	205	60	80	43	55	54	...	...	(120)[c]	(55.9)[c]
IV	101	72[a]	135	55[a]	...	...	153	68	105[b]	62.5[b]
	101	55	70	52	...	...	...	...	(107)[c]	(62.3)[c]
	...	...	71	73	...	...	...	...		
II*	210	60	45	70	70	45	...	...	108[b]	58.3[b]
									(124)[c]	(60.2)[c]
V	150	>330	...	...	60	>420			105[b]	375[b]
Averages	126[b,d]	52[b,d]	79[b]	50[b,d]	77[b,d]	43[b,d]	153[b]	68[b]		
	(127)[c,d]	(53)[c,d]	(80)[c,d]	(50)[c,d]	(78)[c,d]	(47)[c,d]	(157)[c]	(58)[c]		

[a] No acid was administered. In all other runs 50 cc. of 0.1 *N* hydrochloric acid was administered with four enteric coated tablets barium sulfate following an overnight fast.
[b] Unadjusted averages which do not reflect the fact that the dogs were not balanced over the lots.
[c] Adjusted averages.
[d] Averages do not include values of tablet lot V.

TABLE II.—PROPERTIES OF THE COMPRESSED AND SUBCOATED TABLETS BARIUM SULFATE, 3 GR. BEFORE ENTERIC COATING

	Compressed Tablet		Subcoated Tablet				
			Av. Thickness, inches				
Lots of Enteric Coated Tablets	Av. Wt., Gm.	Punch Size	Edge	Side	Av. Wt. Gm.	Av. Volume cu. mm.	Av. Disintegration Time[a] ± S. E.,[b] Min.
I	0.426	3/8 inch oval	0.407	0.207	0.542	301	4.8 ± 0.2
II, III, and V	0.437	3/8 inch oval	0.403	0.214	0.543	305	8.8 ± 0.3
IV	0.421	3/8 inch oval	0.415	0.218	0.606	319	5.8 ± 0.1

[a] Average disintegration time of the subcoated tablets in artificial intestinal fluid, pH 6.9, using the U. S. P. apparatus. The end point was taken when 99 to 100% of the entire tablet was through the screen of the disintegration apparatus. A total of 12 tablets was used for each determination.
[b] Standard error of the average.

TABLE III.—SUMMARY OF IN VIVO AND IN VITRO DISINTEGRATION TIMES AND OTHER DATA FOR THE LOTS OF ENTERIC COATED TABLETS TESTED

	Av. Disintegration Time, Min.		Av. Wt. Applied Per Tablet During Enteric Coating, mg.		Av. Amt. of Coating ± Standard Error			
							Thickness	
Lot	In Vitro $\bar{T}i \pm S_{\bar{T}i}$[a]	In Vivo $\bar{T} \pm S_{\bar{T}}$[b]	Enteric Substance	Dusting Powder	Volume, cu. mm.	Wt., mg.	Edge, inches × 10^3	Side, inches × 10^3
I	15.1 ± 0.3	32.2 ± 2.6	25	91	40.0 ± 1.2	81 ± 3.5	8.8 ± 0.8	7.6 ± 0.5
II	30.1 ± 0.9	45.2 ± 1.7	15	89	39.2 ± 2.4	96 ± 3.8	7.5 ± 0.3	7.6 ± 0.4
III	43.8 ± 0.6	54.0 ± 3.7	12	57	34.2 ± 1.5	73 ± 3.0	4.6 ± 0.3	6.3 ± 0.4
IV	58.2 ± 1.5	62.5 ± 3.9	21	79	44.1 ± 1.9	82 ± 5.6	6.4 ± 0.3	6.4 ± 0.5
II*	59.8 ± 2.2	58.3 ± 7.3	Same as batch II except tablets were stored in sealed flint glass bottles for one month at 47°.					
V	[c]	>375	15	108	47.5 ± 2.5	102 ± 2.6	9.3 ± 0.3	7.6 ± 1.0

[a] Average ± the standard error of the average disintegration time in artificial intestinal fluid, pH 6.9, at 37 ± 2°.
[b] Average ± the standard error of the average disintegration time in the dog's intestine.
[c] Enteric coated tablet Lot V did not completely disintegrate in artificial intestinal fluid, pH 6.9, as did the other lots of tablets. After from fifty to ninety minutes the coatings were broken. After three hours most of the coating and part of the tablets were still on the screen. Five tablets were 90 to 95% through the screen after five hours and one tablet about 50% through the screen after six hours.

pH 6.9, described above at 37 ± 2°, using the same U. S. P. apparatus. The end-point was taken when the whole tablet was 99 to 100% through the screen of the basket. The arithmetical average disintegration time ($\bar{T}i$) of 12 tablets and the standard error of the average ($S_{\bar{T}i}$) for each lot of enteric coated tablets is recorded in Table III. It is noteworthy that the range of *in vitro* disintegration times of each of lots I, II, II,* III, and IV were very small as evidenced by the small standard errors shown in Table III.

The corresponding subcoated tablets were tested by putting them directly into the artificial intestinal fluid, pH 6.9, at 37 ± 2° using the U. S. P. apparatus. The average disintegration times and the standard errors of the averages are shown in Table II.

The average disintegration time of each enteric coating in artificial intestinal fluid, pH 6.9, was

TABLE IV.—IN VITRO RESISTANCE OF ENTERIC COATINGS TO SIMULATED GASTRIC FLUID, pH 1.2, U. S. P. XV AND TO WATER

	Av. Resistance Time ± Standard Error, (in Min.)	
	Simulated Gastric Fluid U. S. P.[a]	Deionized Water[b]
I	285 ± 26[c]	169 ± 2[e]
II	187 ± 6[d]	172 ± 2[d]
III	1 coating attacked after 420 minutes 2 coatings attacked after 470 minutes 3 coatings unaffected after 480 minutes	300 ± 13[d]
IV	187 ± 13[c]	...
V	240[d]	5 coatings attacked after 240 minutes 1 coating unaffected after 360 minutes

[a] Average ± standard error to coating slightly attacked end point in simulated gastric fluid, pH 1.2, U. S. P. XV at 37 ± 2°. The coating slightly attacked end point is defined as that end point where the coating is slightly cracked but the tablet itself shows no distinct evidence of disintegrating or dissolving.

[b] Average ± standard error to coating broken end point in deionized water at 37 ± 2°. The coating broken end point is defined as that end point where the coating has broken through sufficiently so that disintegration or solution of the tablet itself has begun.

[c] Based on 12 tablets.

[d] Based on 6 tablets.

[e] Based on 9 tablets.

assumed to be the difference in the average disintegration time of the whole enteric coated tablet and the average disintegration time of the subcoated tablet.

Some *in vitro* tests were carried out to estimate the resistance of the enteric coatings to simulated gastric fluid (pH 1.2) U. S. P. XV and to deionized water. These tests were also carried out with the U. S. P. apparatus and with the fluids at a temperature of 37 ± 2°. It was usually extremely difficult to obtain an accurate end-point in such tests since after the coating cracked or broke open the pieces usually stayed on the screen for a long time and the enteric coating itself never disintegrated to the point where it would pass through the screen. The most accurate end-points obtainable were those when the coatings were first attacked. The results obtained and a description of the end-points are shown in Table IV.

DISCUSSION AND RESULTS

Correlation of *In Vivo* with *In Vitro* Disintegration Times.—In Fig. 1 the average intestinal disintegration time in the dog is plotted against the average disintegration time in artificial intestinal fluid, pH 6.9. Curve "A" represents the enteric coating only and curve "B" the whole enteric coated tablet. For both curves "A" and "B" the same ordinates were used, but in curve "A" the abscissas represent the *in vitro* disintegration times of the enteric coating only. In curve "B" the abscissas represent the *in vitro* disintegration times of the whole tablet. Curve "C" represents the results that would be expected if the *in vivo* disintegration time equalled the *in vitro* disintegration time.

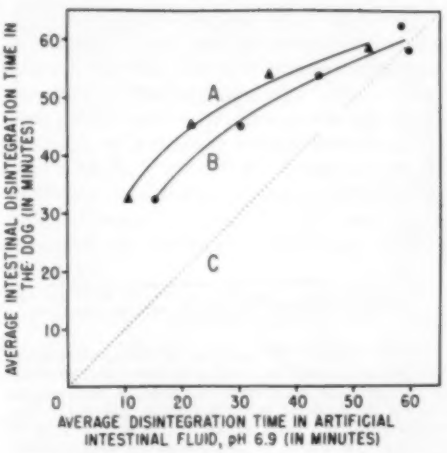

Fig. 1.—Curve "A" is a plot of average intestinal disintegration time in the dog against average disintegration time of the enteric coating in artificial intestinal fluid, pH 6.9 (the latter value was obtained by subtracting the average disintegration time of the subcoated tablet from the average disintegration time of the whole enteric coated tablet). Curve "B" is a plot of average intestinal disintegration time in the dog against average disintegration time of the whole enteric coated tablet in artificial intestinal fluid, pH 6.9. Curve "C" represents the results that would be expected if the *in vivo* disintegration time equalled the *in vitro* disintegration time.

In Fig. 2 the average *in vivo* disintegration time is plotted against the logarithm of the average *in vitro* disintegration time in artificial intestinal fluid, pH 6.9. Line "A" represents the enteric coatings only and line "B" the whole enteric coated tablet. Similarly to Fig. 1, the ordinates for lines "A" and "B" in Fig. 2 were the same but the abscissas differed. Curve "C" represents the results that would be expected with this method of plotting if the *in vivo* disintegration time equalled the *in vitro* disintegration time. The regression equations for the lines "A" and "B" in Fig. 2 are shown beneath the figure. It should be emphasized that these apply only for the dog under the conditions studied and only over the range of disintegration times of tablet lots I, II, II,* III, and IV in the artificial intestinal fluid, pH 6.9. The intestinal disintegration time averages used in Figs. 1 and 2 are unadjusted for lot and dog irregularities.

Lack of Correlation Between the Stomach Retention Time and the Intestinal Disintegration Time.—If the enteric coatings were attacked during the period that they were in the stomach of the dog then one would expect that the tablets would disintegrate at a faster rate in the intestines. Under these conditions one would expect a negative correlation between stomach retention time and intestinal disintegration time. The correlation coefficient between the 27 pairs of values of S and T in Table I is only 0.15 which is not statistically significant at the 5% level. It is noted, however, that when comparisons are made for a particular dog and lot where there is more than one determination, a large

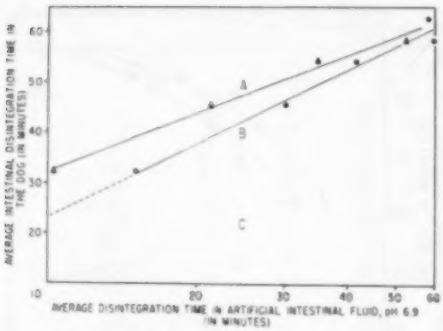

Fig. 2.—Curve "A" is a plot of average intestinal disintegration time in the dog against the logarithm of the average disintegration time of the enteric coating in artificial intestinal fluid, pH 6.9. The equation of the "least squares" line is: *In Vivo* = 37.6 log (*In Vitro*) − 5.3. The correlation coefficient is 0.996 and the standard deviation of scatter is 1.3 minutes. Curve "B" is a plot of average intestinal disintegration time in the dog against the logarithm of the average disintegration time of the whole enteric coated tablet in artificial intestinal fluid, pH 6.9. The equation of the "least squares" line is: *In Vivo* = 47.9 log (*In Vitro*) − 24.7. The correlation coefficient is 0.989 and the standard deviation of scatter is 2.1 minutes. Curve "C" represents the results that would be expected with this method of plotting if the *in vivo* disintegration time equalled the *in vitro* disintegration time.

increase in stomach retention time is sometimes associated with a decrease in intestinal disintegration time. To measure this association, the "between duplicates" correlation coefficient is calculated to be −0.31, based on 11 degrees of freedom, which is also not significant at the 5% level.

When dog to dog and lot to lot differences are ignored there is no correlation between stomach retention time and intestinal disintegration time of the enteric coated tablets. Also, when the influence of dogs and lots is eliminated, there is no significant correlation between these two variables. Hence it may be concluded that the enteric coatings were not attacked in the dogs' stomachs, during the retention times shown in Table I, to a degree which significantly influenced the intestinal disintegration time of the tablets.

Intestinal Disintegration Times in the Dog.—An analysis of variance was carried out on the intestinal disintegration times of tablet lots I, II, III, IV, and II* as shown in Table V. There is a significant difference among lots in average disintegration times (due to the low averages for lots I and II), but there is no significant difference among dogs. The dogs × lots interaction mean square was not significantly greater than the mean square between duplicates (P = 0.10). For the method of analysis, see Snedecor (11).

The unadjusted averages in Table I do not reflect the fact that the dogs were not balanced over the lots. Thus, the average of sixty-eight minutes for dog No. 4 is high because that dog was used only for lot IV which has the highest disintegration time in this group of lots. Adjusted averages are also shown in Table I and these averages do reflect that the dogs were not balanced over the lots.

Tablet lot V, the only lot without a clear-cut disintegration time in artificial fluid, pH 6.9, did not disintegrate in the intestinal tract during the five and one-half hour observation period in one dog nor during the seven-hour observation period in a second dog. These tablets did show broken coatings after fifty to ninety minutes and were practically all disintegrated after five hours in the artificial intestinal fluid.

Five of our lots of tablets had average disintegration times in the range thirty to sixty minutes in artificial intestinal fluid, pH 6.9, after a two-hour period in simulated gastric fluid. These tablets all had average disintegration times in the dog's intestine from forty-five to sixty-two minutes. It is realized that disintegration time observed roentgenographically does not guarantee intestinal absorption of the contained drug (12, 13). However, these data do indicate that similar experiments coupled with blood level or urinary excretion studies should be carried out in the human being before definite new standards, such as proposed by Chapman, *et al.* (4–7), concerning disintegration tests for enteric coated tablets are established.

Stomach Retention Times of Enteric Coated Tablets in the Dog.—An analysis of variance was carried out on the stomach retention times of lots I, II, III, IV, and II.* The results of this analysis are shown in Table VI. There were no significant differences among lots; this would be expected since the coated tablets were all approximately the same size. There were significant differences among dogs, but the interaction between dogs and lots was not significant (P = 0.19).

***In Vivo* Resistance of the Enteric Coated Tablets to Stomach Conditions and *In Vitro* Resistance of the Same Lots of Tablets to Simulated Gastric Fluid U. S. P. XV.**—As stated previously, in the *in vivo* resistance studies the dogs were fed thirty minutes prior to the administration of the enteric coated tablets. Under these conditions tablets of lots I, II, and III remained intact in the stomachs of the dogs for a period greater than seven and one-half hours at which time the tests were terminated. One

TABLE V —ANALYSIS OF VARIANCE OF INTESTINAL DISINTEGRATION TIMES OF FIVE LOTS OF ENTERIC COATED TABLETS IN FOUR DOGS

Source of Variation	d.f.	S.S.	M.S.
Dogs	3	154	51
Lots	4	2758	690[a]
Interaction (dogs × lots)	7	761	109
Between duplicates	12	560	47

[a] Significant at P = 0.001.

TABLE VI —ANALYSIS OF VARIANCE OF STOMACH RETENTION TIMES OF FIVE LOTS OF ENTERIC COATED TABLETS IN FOUR DOGS

Source of Variation	d.f.	S.S.	M.S.
Dogs	3	16,118	5,373[a]
Lots	4	4,229	1,057
Interaction (dogs × lots)	7	12,460	1,780
Between duplicates	12	11,642	970

[a] Significant at P = 0.05.

experiment was carried out for each of lots II and III in dog No. 1 and two runs were carried out for lot I in dogs No. 1 and No. 3. During the testing of lot IV it was found that even when the dogs were not fed prior to the administration of the tablets, that in some cases the tablets remained in the stomachs of the dogs for longer periods of time than usual. The results obtained with lot IV are summarized in Table VII. Lot IV exhibited variable resistance to stomach conditions in starved dogs.

The correlation between the resistance of the enteric coated tablets to stomach conditions in the dog and the *in vitro* resistance of the coatings to simulated gastric fluid U. S. P. XV does not appear too good. The only lot which correlates well is lot III which resisted simulated gastric fluid for from seven to greater than eight hours and which resisted stomach conditions in the fed dogs for greater than seven and one-half hours. However, for lots I, II, and IV the time to the coating slightly attacked end-point in simulated gastric fluid was considerably less than the resistance time of the same lots in the stomachs of dogs. We do have data, which will be published in the future, which indicate that there is a good correlation between resistance time of enteric coated tablets in the stomach of the human being and the time to the coating cracked open end-point in simulated gastric fluid U. S. P. XV.

TABLE VII—RESISTANCE OF TABLET LOT IV TO STOMACH CONTENTS OF STARVED DOGS

Dog	Acid or no acid Administered	Time Experiment Terminated, Hours	State of Tablets and Remarks
No 1	Acid	6.5	Disintegrated $5^1/_2$ to $6^1/_2$ hr.
No 4	Acid	6.0	Tablets intact
No 4	Acid	2.75	Tablets intact
No 4	No acid	5.75	Tablets intact
No. 4	No acid	6.0	Disintegrated 6 hr. pH of stomach contents was 5.3

SUMMARY AND CONCLUSIONS

1. The stomach retention times and intestinal disintegration times of six different lots of enteric coated tablets were determined roentgenographically in the dog.

2. The resistance of the enteric coatings to simulated gastric fluid U. S. P. and the disintegration times of the whole enteric coated tablets and of the subcoated tablets in artificial intestinal fluid, pH 6.9, are reported.

3. Graphs and equations are presented which express the observed relationship between the average intestinal disintegration time in the dog and the average disintegration time of the whole enteric coated tablet or of the enteric coating only in the artificial intestinal fluid over a range of disintegration times of approximately fifteen to sixty minutes.

4. When dog to dog and lot to lot differences were ignored there was no correlation between stomach retention time and intestinal disintegration time of the enteric coated tablets. Also, when the influence of dogs and lots was eliminated, there was no significant correlation between the two variables. Hence, it was concluded that the enteric coatings were not attacked in the starved dogs' stomachs to a degree which significantly influenced the intestinal disintegration time of the tablets.

5. There were no significant differences among lots in average stomach retention time which would be expected since the tablets were all approximately the same size. There were significant differences among dogs in average stomach retention time, but the interaction between dogs and lots was not significant.

6. There were significant differences among lots but there were no significant differences among dogs in average intestinal disintegration time. The interaction between dogs and lots was not significant. One lot of tablets, not included in the analyses, which did not have a definite disintegration time in artificial intestinal fluid, pH 6.9, failed to disintegrate after greater than three hundred and thirty minutes in the intestinal tract of one dog and after four hundred and twenty minutes in a second dog.

7. The methods presented are useful methods to screen new enteric coatings and to check the effects of various storage conditions on the disintegration time of enteric coated tablets.

8. It is recommended that similar experiments coupled with blood level studies be carried out in the human being before definite new standards concerning disintegration tests for enteric coated products be established.

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A Note on the Assay of Digitoxin Preparations*

By DANIEL BANES, ALBERT E. H. HOUK, and JACOB WOLFF

THE METHOD recently adopted for the analysis of digitoxin preparations by the Association of Official Agricultural Chemists (1) modifies U. S. P. XV assay procedures for the drug (2) in several respects. It simplifies preparation of samples, chromatographic columns, and solvents, eliminates the isooctane wash, requires a smaller volume of eluent, and provides a test for detecting extraneous digitoxosides.

We have analyzed several samples of digitoxin powders and twenty-one lots of digitoxin tablets by both the modified method and the official assay procedures. Results of these analyses (Table I) show that the two methods yield concordant assay values.

To test the efficiency of the chromatographic column in separating digitoxin from gitoxin and more polar digitoxosides, we added varying quantities of gitoxin to two of the crystalline powders, and reanalyzed the mixtures. Digitoxin was recovered in the first 200 ml. of eluate. The following 50-ml. portion contained less than 0.4% of the total digitoxin when determined by means of the alkaline picrate reagent. All of the added gitoxin was recovered in the proposed test for other digitoxosides (1). When the digitoxin eluates from tablet samples were similarly tested, the last 50 ml. of solution were practically devoid of digitoxin. The prescribed volume of 250 ml. includes a safety factor to ensure complete separation of digitoxin from gitoxin and more polar digitoxosides.

TABLE I.—ANALYSIS OF DIGITOXIN PREPARATIONS

Sample	Description	Digitoxin Found, Per Cent of Declared: Modified Method	Official Method
D-1	Crystalline powder	96.5	95.9
D-1a	D-1 plus 4% added gitoxin	95.7	...
D-1b	D-1 plus 10% added gitoxin	96.3	...
D-2	Crystalline powder	89.5	87.6
D-3	Crystalline powder	86.5	84.2
D-4	Crystalline powder	83.3	82.4
D-4a	D-4 plus 4% gitoxin added	82.6	...
D-4b	D-4 plus 10% gitoxin added	82.1	...
	Tablet (mg.)		
T-1	0.1	105.6	103.0
T-2	0.1	103.9	100.7
T-3	0.2	99.4	95.3
T-4	0.1	97.2	95.9
T-5	0.2	93.5	91.0
T-6	0.1	93.3	92.8
T-7	0.1	92.5	88.9
T-8	0.2	92.0	91.7
T-9	0.1	91.9	90.0
T-10	0.2	91.5	94.0
T-11	0.2	90.3	88.7
T-12	0.1	88.4	86.3
T-13	0.1	88.3	87.6
T-14	0.1	86.5	85.2
T-15	0.2	83.8	85.7
T-16	0.15	83.5	85.0
T-17	0.2	79.3	80.1
T-18	0.1	77.3	77.2
T-19	0.1	60.0	56.3
T-20	0.1	45.5	46.6
T-21	0.1	113.3	113.0

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* Received December 27, 1957, from the Division of Pharmaceutical Chemistry, Bureau of Biological and Physical Sciences, U. S. Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

A Note on a Simple Small Animal Respirometer*

By S. T. COKER

VARIOUS METHODS have been used to record respiration in small animals (1–5). Most of the existing methods are not satisfactory for animals as small as rats or lack adequate sensitivity. Also, many of these methods are rather complex, or involve an elaborate system of valves. The respirometer described below is based on the principle originally used by Gaddum (1) for recording respiration in rabbits. A modification of Gaddum's procedure by Haley (2) is similar to the one described below except a different system of valves is used. This led to the development of a small, simple respirometer which could be built completely and inexpensively in any laboratory and have sufficient sensitivity to record accurately respiration of rats as well as larger animals with proper modifications as to chamber volume and diameter of the air inlets and outlets. The apparatus is quite suitable for use in laboratory teaching as well as drug screening on rats.

The materials needed are: Plexiglas tubing one inch outside diameter by two inches in length for the chamber, and $^1/_8$-inch outside diameter tubing for the necessary connections, a No. 4 rubber stopper, rubber tubing, medium rubber dam, ethylene dichloride for cementing the Plexiglas, screw clamp, tracheal cannula, sensitive tambour, and a recording lever (Fig. 1, *A*).

* Received February 12, 1958, from the Department of Pharmacology, University of Kansas City, School of Pharmacy, Mo.
The author is indebted to John R. Lyon, senior laboratory assistant, for his valuable technical assistance.

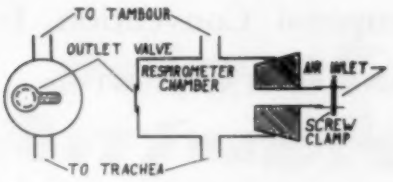

Fig. 1-A

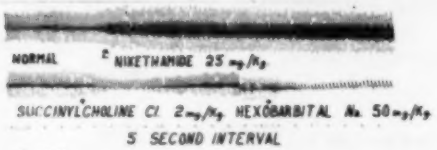

Fig. 1-B

Fig. 1,*A*.—Assembled respirometer.
Fig. 1,*B*.—Respiratory effects produced by niketh-amide, succinlycholine, and hexobarbital administered i. v. to a rat anesthetized with pentobarbital Na, 40 mg./Kg. i. p.

The principle of the respirometer is that for a short time during inspiration there is a fall in pressure inside the chamber which the tambour records. When the animal inspires, the air outlet valve closes and air enters the chamber at the opposite end through a restricted inlet controlled by the screw clamp. During expiration the air escapes through the 3–4 mm. air outlet which is controlled by a rubber dam valve. Greater recording amplitude is obtained if the lever is connected to the center of the tambour by a thread rather than allowed to rest directly on a lever support.

Dead air space should be held to a minimum by keeping the connecting tubes as short as possible. The rubber stopper allows free access to the inside of the chamber for charging with soda lime granules and for cleaning. Experiments on rats have been extended for as long as two hours and there was no evidence of oxygen deficiency or carbon dioxide accumulation. The effects of some common respiratory stimulants and depressants are shown (Fig. 1, *B*).

SUMMARY

A simple, inexpensive and easily constructed small animal respirometer suited especially for rats is described. It has been found useful in studying central nervous system and respiratory stimulants and depressants as well as muscle relaxants.

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Book Notices

Tracer Applications for the Study of Organic Reactions. By John G. Burr, Jr. Interscience Publishers, Inc., New York, 1957. x + 291 pp. 15 x 23 cm. Price $7.50.

In this book the author has provided a reasonably comprehensive survey of tracer applications in organic chemistry and some generalization of the principles of isotopic tracer use. Sufficient background information about reaction mechanisms is given to enable the reader to judge the advantages of each method described. The book should stimulate the use of isotopes by organic chemists. It is documented with references to the original literature and is provided with adequate author and subject indexes.

Chemotherapy and the Central Nervous System. By Henry McIlwain. Little, Brown & Company, Boston, 1957. vii + 327 pp. 15.5 x 23.5 cm. Price $10.

This book presents the development of methods and principles in chemotherapy and the application of chemotherapy in the treatment of mental disorders. The nature and results of chemotherapeutic trial and the structure and dosage for each chemotherapeutic agent are included. References and author and subject indexes are appended.

Trace Analysis. Edited by John H. Yoe and Henry J. Koch, Jr. John Wiley and Sons, Inc., Publishers, New York, 1957. xiii + 672 pp. 15 x 23 cm. Price $12.

This work is based on a symposium on trace analyses held at the New York Academy of Medicine in 1955 and includes chapters written by twenty-four contributors. Methodology, instrumentation, separations, concentrations, and contamination hazards in dealing with sub-microgram quantities are discussed. Each of the twenty-four chapters is followed by a discussion including pertinent comments made from the floor at the time of the symposium. The papers deal with the theory, application, sensitivity, precision, accuracy, sources of error, advantages, and limitations of the various methods and techniques used in trace analysis. The book is documented throughout including select references to books on specific subjects.

Pharmacology and Therapeutics. 3rd ed. By Arthur Grollman. Lea and Febiger, Philadelphia, 1958. 1034 pp. 15 x 23 cm. Price $12.50.

This standard textbook represents a revision of Volume II which was published in 1954.

The United States Pharmacopœial Convention, Inc.

EIGHTH ANNUAL FINANCIAL STATEMENT

Based upon the Report of the Auditor for the year ended December 31, 1957

STATEMENT OF INCOME AND EXPENSE

Income:			
Sales of pharmacopeias:			
Collections		$62,263.30	
Less—Decrease in accounts receivable		5,200.25	
			$ 57,063.05
Sales of reference standards:			
Collections		34,392.00	
Add—Increase in accounts receivable and adjustment of prior years' collections		1,970.24	
			36,362.24
Interest on investments, less amortization of bond premiums of $22.65			6,839.85
Interest on savings deposits			6,330.21
Use of text by others			615.00
Miscellaneous			107.60
Total Income			$107,317.95
Less—Expense:			
Revision		$96,013.29	
Add—Decrease in inventory		81.64	
			$ 96,094.93
Administration			7,826.04
Publications		836.17	
Add—Decrease in inventory		14,829.27	
			15,665.44
Headquarters		5,134.32	
Add—Depreciation expense:			
Building	$2,430.77		
Furnishings & Equipment	3,802.75	6,233.52	
			11,367.84
Total Expense			$130,954.25
Excess of Expense over Income			$ 23,636.30

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

Receipts:		
Sales of pharmacopeias	$ 62,263.30	
Sales of reference standards	34,392.00	
Interest on investments	6,862.50	
Interest on savings deposits	5,759.20	
Use of text by others	615.00	
Miscellaneous	107.60	
Total Receipts	$109,999.60	

Expense:

	Revision	Administration	Publications	Headquarters	Furnishings and Equipment	Total
Expense:						
Printing & Binding	.....	.....	$667.15	.....	.....	$ 667.15
Salaries	$55,411.01	$5,369.99	...	$1,650.00	.....	62,431.00
Meetings	4,887.54	1,574.89	...	.....	.....	6,462.43
Supplies	1,714.55	144.52	...	.....	.....	1,859.07
Postage & Telegrams	1,206.84	26.54	...	.....	.....	1,233.38
Utilities	.....	.....	...	2,181.11	.....	2,181.11
Repairs & Maintenance	.....	.....	...	1,068.95	.....	1,068.95
General	32,793.35*	710.10	169.02	.....	.....	33,672.47
Insurance & Taxes	.....	.....	...	234.26	.....	234.26
Furnishings & Equipment	.....	.....	...	.....	6,651.33	6,651.33
Total Expense	$96,013.29	$7,826.04	$836.17	$5,134.32	$6,651.33	$116,461.15

*Exhibits & Promotion	$ 5,098.00
Reference Standards (supplies only)	7,658.98
Research Assistants	11,500.00
Retirement & Social Security	7,761.11
Miscellaneous	775.26
	$32,793.35

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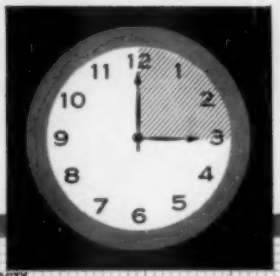

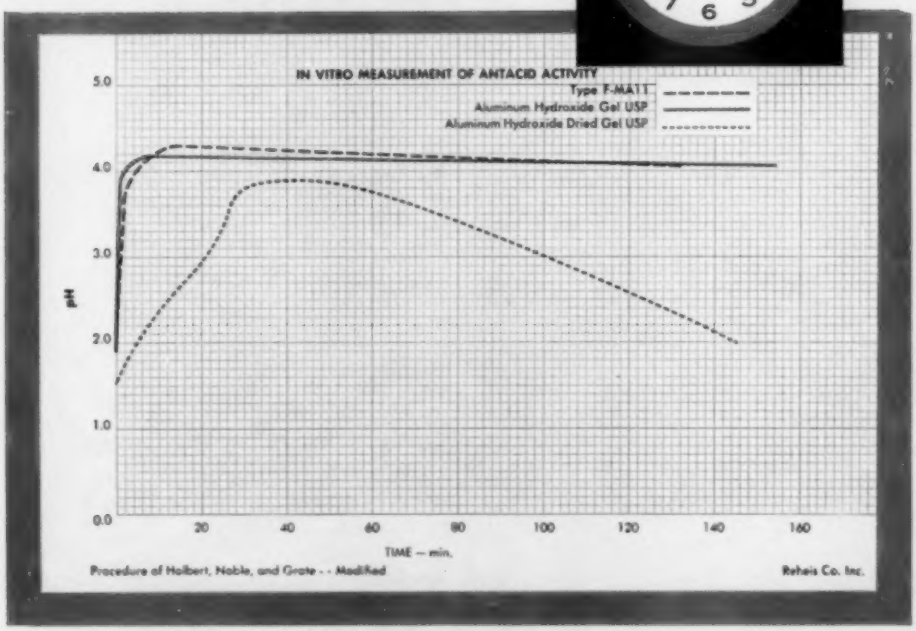

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